Regulation of the synthesis and activity of the STP1 monosaccharide transporter in *Arabidopsis thaliana*

PhD

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Abstract

In plants the effective partitioning of carbon and nitrogen assimilates between source and sink tissues is essential for growth and development. Sugar signalling is important in modulating the expression of genes associated with the distribution, utilisation and storage of assimilates. Thus the regulation of sugar transporters is pivotal to the allocation of carbohydrates throughout the plant. *At*STP1 (Sugar Transport Protein 1) accounts for approximately 60% of 3-O-methyl glucose uptake activity in *Arabidopsis* seedlings and therefore is the major monosaccharide transporter in *Arabidopsis thaliana*. Characterisation of *AtSTP1* gene expression and transport activity in plantlets grown *in vitro* in continuous light and in a diurnal growth regime reveals regulation by light, glucose and mechanical stimulation. A rapid accumulation of *AtSTP1* transcripts upon transfer to the dark is observed in all growth regimes. This appears to be a novel feature in sugar transporter regulation. In light-grown plantlets, dark-induced *AtSTP1* transcripts diminish within 1 h of treatment with exogenous 3 mM D-glucose or white light. Furthermore, a 5 min pulse of white light results in a marked repression of dark-induced *AtSTP1* transcripts, which is indicative of sugar-independent regulation. The diurnal expression pattern, and the sugar response of the *AtSTP1* gene is complex in plantlets entrained to a L12 h:D12 h regime. The expression of the *AtSTP1* gene is different in the shoots and roots; furthermore in the shoots *AtSTP1* transcripts display circadian rhythm upon transfer of plantlets to continuous light. It appears that at least three signalling pathways interact in the regulation of monosaccharide transport in *Arabidopsis*. These results are discussed in terms of possible physiological functions for *At*STP1, including a role in the retrieval of sugars derived from the cell wall.

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Abbreviations

(v/v)	volume:volume ratio
(w/v)	weight:volume ratio
°C	degree Celsius
µM	micromolar
A	absorbance
ABA	abscisic acid
ATP	adenosine 5'-triphosphate
B	blue light
bp	base pairs
BSA	bovine serum albumin
C	carbon
C-terminal	carboxy terminal
Ca^{2+}	calcium ion
CAB	chlorophyll a/b binding protein
cDNA	complementary DNA
Ci	Curie
Col0	*Arabidopsis thaliana* ecotype Columbia 0
cpm	counts per minute
cR	continuous red light
cwINV	cell wall invertase
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
dNTPs	deoxynucleoside triphosphates
dsDNA	Double stranded DNA
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FADH	Flavin adenine dinucleotide (reduced form)
Frc	fructose
FR	far red light
Gal	galacose
Glc	glucose
H^+	proton
HCL	hydrogen chloride
HEPES	N-(2-hydroxyethyl)-N-(2-ethanesulphonic acid)
Hyg^r	hygromycin resistance
HXT	Yeast hexose transporter protein family
Hxk	hexokinase
K^+	potassium ion
Kbp	kilobase pairs
kDa	kilodalton
luc^+	luciferase reporter gene
LB	Luria-Bertani medium
Man	mannose
mRNA	messenger RNA
mM	millimolar
MS	Murashige and Skoog medium
N	nitrogen
N-terminal	amino terminal
Na^+	sodium ion
NaOH	sodium hydroxide
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced form)

OD	optical density
PCR	polymerase chain reaction
R	red light
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
AtSTP	*Arabidopsis thaliana* sugar transport protein family
STP1	Sugar Transport Protein 1
SNF1	Sucrose Non-Fermenting-1-Protein kinase
Suc	sucrose
TAE	tris-acetate-EDTA
TBE	tris-borate-EDTA
TE	tris-EDTA
T-DNA	transfer DNA
Ti	tumour inducing
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
3'UTR	3' untranslated region
UV	ultraviolet
Ws	*Arabidopsis thaliana* ecotype Wassilewskija
V	volt
Xyl	xylose
Yeast	*Saccharomyces cerevisiae*

CHAPTER ONE: Introduction

1.1 Assimilate partitioning- the importance of sugar transporters

Different organs of the plant have diverse functions and requirements. An important role of source leaves is the synthesis of energy-rich carbon assimilates, whereas heterotrophic sink organs are dependent on the import of assimilates. The flux of assimilates between source and sink tissue is controlled to optimise the supply and utilisation of carbon and nitrogen resources. Generally, the utilisation of sugars in growing sinks is dependent on the simultaneous provision of amino acids. The integration of carbon and nitrogen metabolism prevents excess accumulation of carbon skeletons or amino acids. Consequently, many of the genes associated with source and sink metabolism are regulated in a co-ordinated manner by both sugars and intermediates of the nitrogen assimilation pathway (Martin *et al.,* 2002; Coruzzi and Zhou, 2001; Paul and Foyer, 2001; Lewis *et al.,* 2000).

Generally, when carbon skeletons are abundant and internal levels of organic nitrogen are low the genes for nitrogen assimilation and remobilisation are expressed, as well as sugar-responsive genes associated with carbohydrate biosynthesis and storage. When the levels of photosynthate are low or internal levels of organic nitrogen are high the expression of genes for photosynthesis, reserve mobilisation and nitrate metabolism are expressed (Smeekens, 2000; Koch, 1996).

Plants are photo-autotrophic organisms that are composed of many heterotrophic tissue systems, which rely on the import of sugars and amino acids. Sugars, amino acids and other solutes are transported from leaf tissue (source), where photosynthetic machinery converts light energy into exportable chemical energy, into the vascular network. Thereafter phloem sap movement, unloading and post-phloem transport deliver assimilates to various heterotrophic sink tissues, such as,

storage sinks (seeds, tubers and fruits) or utilisation sinks (flowers and roots) (Bush *et al.,* 1999). Sugar transport plays an important role in the partitioning of carbon throughout the plant.

In most plants, sucrose is the major photosynthetic product and the carbohydrate used in long distance transport between source and sink tissues (figure 1.1). Sucrose may be transported from source mesophyll cells to the phloem either via symplastic movement or apoplastic H^+-sucrose symporters. The energy for the active uptake of sucrose is generated from the proton motive force of H^+-ATPase activity. The phloem consists of at least two cell types: companion cells (CC) and sieve elements (SE), which are highly modified and interconnected via plasmadesmata. Phloem sap moves through the vascular system by mass flow. At sink tissues, sucrose can be unloaded either directly into sink cells via plasmadesmatal connections or via the apoplast. Subsequent uptake by sink cells from the apoplast may occur by H^+-sucrose symporter activity. Alternatively, sucrose can be hydrolysed to glucose and fructose by extracellular invertases and transported by H^+-monosaccharide transporters (Lalonde *et al.,* 1999; Williams *et al.,* 2000).

One requirement for the apoplastic uptake of glucose is the co-expression of extracellular invertase and monosaccharide transporters (Ehness and Roitsch, 1997). Extra-cellular invertase plays a pivotal role in assimilate partitioning, for it determines whether sink cells are provided with sucrose or glucose and fructose (Roitsch *et al.,* 1995), which in turn can trigger changes in plant development (Sturm and Tang, 1999). For example, in *Vicia faba* seed development sucrose promotes cotyledon expansion and resource accumulation and glucose promotes cell division (Weber *et al.,* 1996).

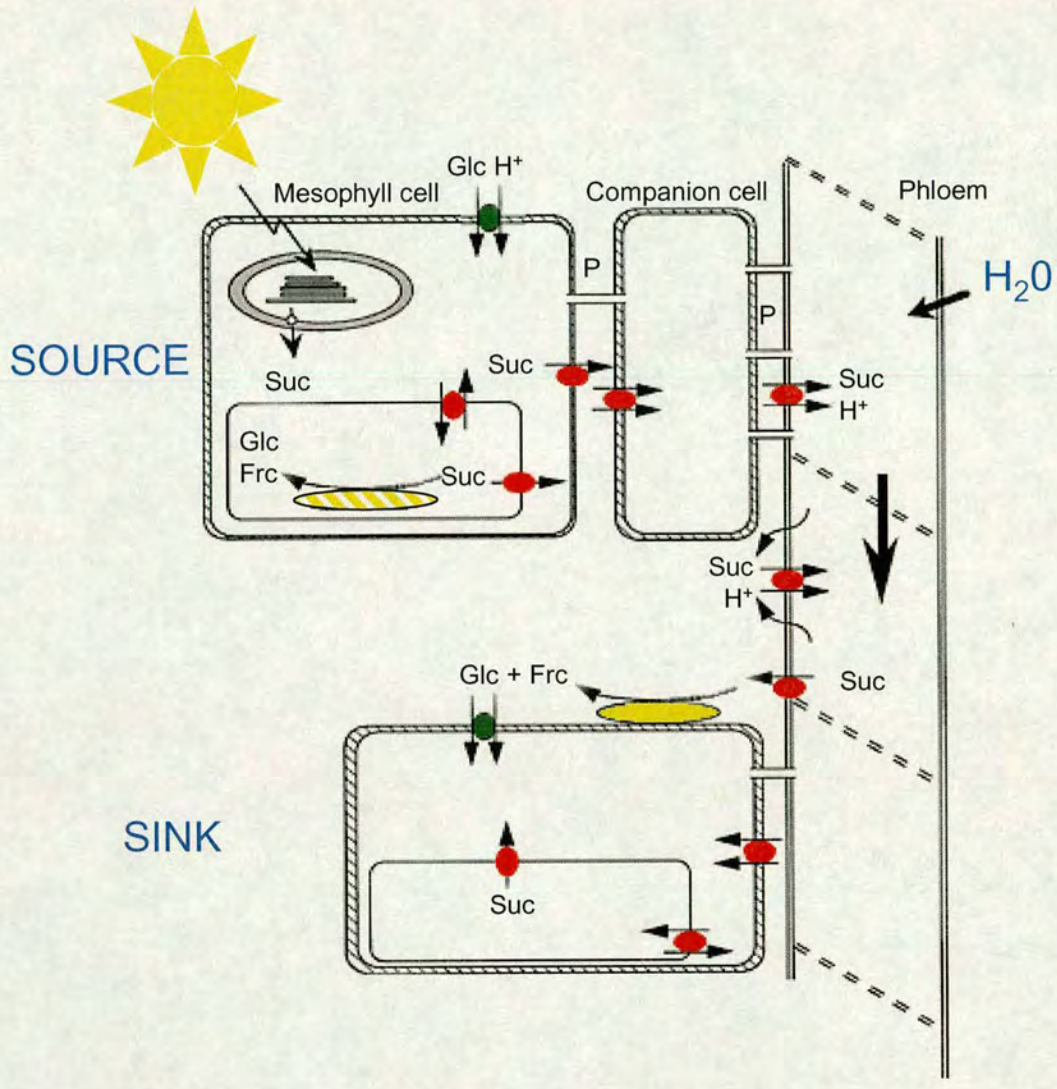

Figure 1.1 Contribution of sugar transporters in the long-distance transport of sugar between source and sink tissues.

Sucrose (Suc) produced from photosynthesis in mesophyll cells (source tissue) can pass to the phloem cell to cell via plasmadesmata (P). In source cells, sucrose may also be hydrolysed to glucose and fructose by cytosolic or vacuolar (stripped yellow circle) invertases; hexose symporters (green, 2 arrows) may function in the retrieval of sugars that leak from source cells. Sucrose may also leave the symplast of cells possibly via sucrose efflux carriers (red circle, single arrow), and can be loaded into companion cells or the phloem by plasma membrane H^+-Suc symporters (red circles, 2 arrows). The passive influx of water into the phloem aids the mass flow of sucrose to sink tissues. A number of sucrose transporters are expressed along the phloem, these may function in the retrieval of sucrose that may leak into neighbouring cells. Sucrose unloading into sink cells may occur sympastically via plasmadesmata, or apoplastically via suc symporters. Alternatively sucrose may be hydrolysed to glucose and fructose by cell wall invertase (yellow circle) and taken up into sink cells via plasma membrane hexose symporters. (Modified from Lalonde *et al.*, 1999)

Extracellular invertases appear to be tightly regulated both transcriptionally and post-transcriptionally by a range of biotic and abiotic factors (Sturm and Tang, 1999; Roitsch, 1999). The importance of the provision of apoplastic hexose for normal plant development is highlighted through the analysis of the *miniature1* mutant of *Zea mays* and antisense inhibition of cell wall invertase (cwINV) synthesis in carrot. The *miniature1* mutant lacks an endosperm-specific cwINV; consequently mutant seeds are small because the endosperm fails to develop (Cheng *et al.,* 1996). In antisense cwINV carrots, the tap root formation is abolished and foliar growth is favoured (Tang *et al.,* 1999).

The distribution of monosaccharide (MST) and disaccharide (DST) transporters throughout the plant is diverse, and is likely to be indicative of the sugar provision (sucrose or hexoses) to specific tissue types. In *Arabidopsis STP4* (MST) mRNA is expressed in root tips (Truernit *et al.,* 1996) and in *Medicago truncatula* the *MST1* gene is expressed in the root elongation zone (Harrison, 1996), which suggests that such cells are provided with hexoses rather than sucrose and that post-phloem transport in the root is likely to occur apoplastically. In elongating cells MST may play a role in the provision of hexoses as precursors of macromolecular synthesis and in the maintenance of osmotic pressure within the cell during elongation (Harrison, 1996). A sucrose transporter (DST) has also been localised to carrot tap roots (Shakya and Sturm, 1998).

Sugar transporters (DST) appear to play an important role in phloem loading, in several species sucrose transporters have been localised to either the sieve element or the companion cells of the phloem; for example, *Plantago major* (Stadler *et al.,* 1995; Gahrtz *et al.,* 1996), *Arabidopsis* (Stadler and Sauer, 1996) and *Solanum* species (Kuln *et al.,* 1996; Weise *et al.,* 2000) including tobacco (Burkle *et al.,* 1998).

The expression of both MST and DST has been reported in developing seeds. The developing embryo is apoplastically isolated from the maternal tissue and therefore is dependent on the apoplastic provision of sugars (Wobus and Weber, 1999a). In *Vicia faba*, MSTs are expressed in regions consisting of mitotically active tissues, where it is likely sugars are used in cell metabolism. Later in embryo development DST are expressed, providing sugars for resource accumulation and storage (Weber *et al.,* 1997). Sucrose transport (DST) is also required upon the mobilisation of storage reserves in germinating *Ricinus communis* seeds (Weig and Komor, 1996).

In several plants, MSTs are expressed in developing pollen grains, during pollen germination and pollen tube growth, and in mature anthers and pollen (Ylstra *et al.,* 1998; Truernit *et al.,* 1996 and Truernit *et al.,* 1999). However, there is overlap in the use of MST and DST between species, for example, sucrose is the preferred carbon source during pollen germination in *Arabidopsis* and tobacco grown *in vitro* (Stadler *et al.,* 1999; Lemoine, 1999), which suggests cwINV activity is not involved.

Given the high number of sugar transporters identified in plants, and the often varied localisation of their expression, it is difficult to assign a precise function and physiological role for each transporter.

1.2 Monosaccharide Transporters

Co-transporters are membrane transport proteins that couple the uptake of substrate to an electrochemical gradient. In most animal cells co-transporters use Na^+- or K^+- ATPase to drive transport, whereas most plant, fungal and bacterial cells couple substrate transport to proton gradients generated by H^+-ATPase (Sze *et al.*, 1999). An important group of H^+-coupled co-transporters are the nutrient

transporters, which in plants play an essential role in the partitioning of assimilates between source and sink tissues.

Many nutrient transporters are members of the major facilitator superfamily (MFS) of transport proteins. Members are from many different origins (prokaryotic and eukaryotic) and catalyse uniport, antiport and symport of substrates. A symporter imports both substrate and H^+, such as transporters localised to the plasma membrane. An antiporter imports the substrate and exports the H^+, such transporters are likely to be responsible for the loading of glucose, Ca^{2+} and Na^+ into vacuoles and thus preventing substrate accumulation in the cytoplasm. Uniporters/ efflux carriers are responsible for facilitated diffusion, whereby specific carriers move molecules across the membrane; the direction of the net flux is down the potential energy gradient (Marger and Saier, 1993; Bush, 1993).

The first eukaryotic H^+-coupled co-transporter gene to be cloned encoded the plasma membrane hexose symporter (HUP1) from *Chlorella kessleri* (Sauer and Tanner, 1989). Cloning of other higher plant monosaccharide transporters was accomplished by heterologous hybridisation of the *HUP1* probe to similar DNA sequences (Sauer *et al.,* 1990b). This method has lead to the identification of more than 26 genes from *Arabidopsis thaliana* and multiple genes from other species (Lalonde *et al.,* 1999). For example, the H^+-hexose transporter STP1, which has been functionally characterised in Yeast as a proton symporter (Sauer *et al*., 1990).

The Sugar Transport Proteins (STPs) are a family of monosaccharide transporters in *Arabidopsis thaliana*. There are at least 14 putative *AtSTP* genes within a family of at least 50 closely related genes (Noiraud *et al.,* 2000). Four *At*STP proteins have been studied in greater detail and appear to have distinct substrate specificity, temporal expression and functions (Table 1.1; and reviewed by Buttner and Sauer,

Table 1.1 The functional characteristics of several plant monosaccharide-H^+ symporters.

Monosaccharide transporters	K_M	Transported substrates	Site of gene expression	Reference
C. *kessleri* HUP1	15 μM Glc	Glc>Frc>Man>Xyl>Gal	-	Sauer *et al.*, 1990
A. *thaliana* STP1	50 μM Glc	Glc>Gal>Man>Frc	leaves, roots, stems, flowers, siliques	Sherson *et al.*, 2000
A. *thaliana* STP2	50 μM Gal	Gal>Xyl>Glc=Man	developing pollen	Truernit *et al.*, 1999
A. *thaliana* STP3	2 mM Glc	Glc>Xyl>Man>Gal	green tissue, and upon wounding	Buttner *et al.*, 2000
A. *thaliana* STP4	15 μM Glc	Gal>Glc>Xyl=Man	root tips, pollen tube, leaves and upon wounding	Truernit *et al.*, 1996

2000). *At*STP1 is the major transporter in the family, accounting for approximately 60% of glucose uptake activity in *Arabidopsis* seedlings. *At*STP1 is active during seed germination and seedlings growth (Sherson *et al.,* 2000). *At*STP2 is found specifically in developing pollen and is thought to take up glucose derived from callose degradation during pollen maturation (Truernit *et al.,* 1999). *AtSTP3* mRNA is found in leaves (Buttner *et al.,* 2000). *AtSTP4* mRNA is induced by wounding and pathogen attack (Truernit *et al.,* 1996).

1.3 Sugar Transport Protein 1 (STP1)

STP1 is a highly hydrophobic protein of 522 amino acids with a predicted molecular mass of 57 kDa. The hydrophobicity profile of STP1 suggests a topology of 12 trans-membrane domains, similar to that of other MFS transporters (figure 1.2). It is thought that the 12 membrane-spanning regions are grouped in two 6 trans-membrane domains separated by a long (63 amino acids) central hydrophilic (charged amino acids) cytoplasmic loop with both the N and C termini located on the cytoplasmic side of the plasma membrane (Sauer and Tanner, 1989; Alomia and Manoil, 1990; Baldwin, 1993).

Functional characterisation of *At*STP1 in *Schizosaccharomyces pombe* (Sauer *et al.,* 1990), *Saccharomyces cerevisiae* (Stolz *et al.,* 1994) and *Xenopus* (Boorer, *et al.,* 1992 and 1994) demonstrated that *At*STP1 is a high-affinity, low-specificity monosaccharide-H^+ symporters. *At*STP1 transports several monosaccharides, including 3-O-methyl-D-glucose, D-glucose, D-xylose, L-arabinose, D-galactose, although D-fructose transport is negligible.

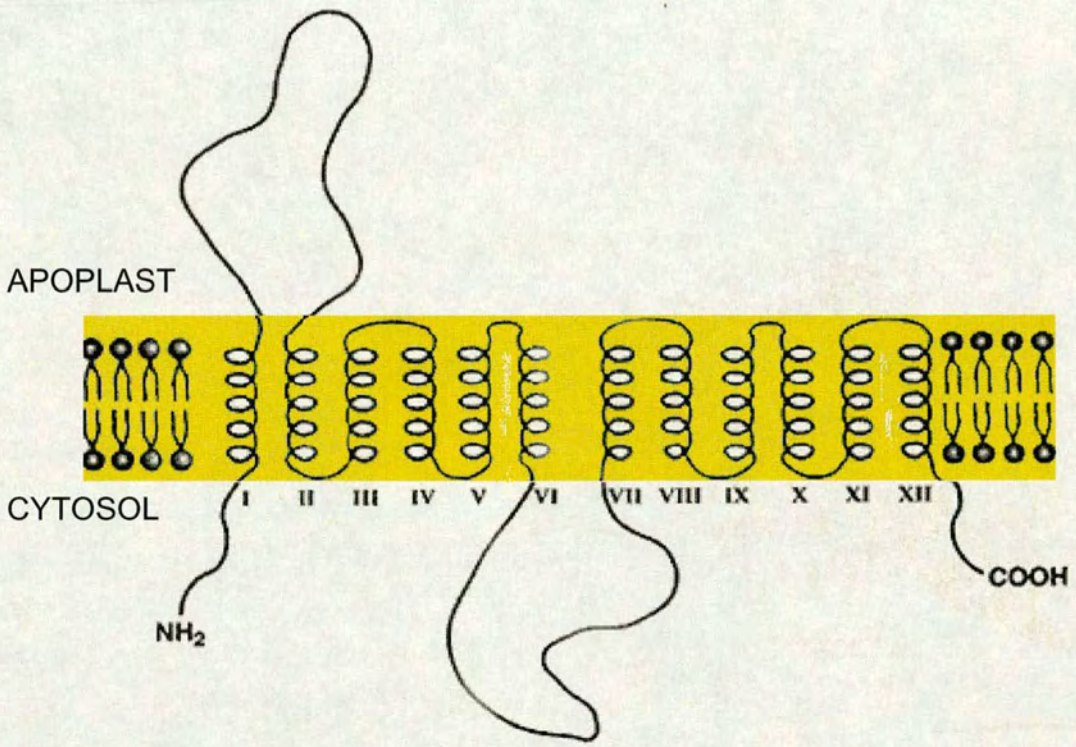

Figure 1.2 **The putative structure of MFS transporters, including STP1 monosaccharide transporter.** (Modified from Barrett *et al.*, 1999)

AtSTP1 mRNA is most abundant in leaves but also found in green pods, flowers, roots and stems (Sauer *et al.,* 1990). More recently, *At*STP1 transport activity has been identified during the germination of seeds and seedlings growth. Furthermore, the substrate specificity of *At*STP1 is similar to the sugar composition of the primary cell wall, which argues for a possible role in cell wall sugar salvage (Sherson *et al.,* 2000). However, no specific function for *At*STP1 has been confirmed to date. Comparison of the rate of D-glucose uptake by wild-type seedlings and *Atstp1* mutant seedlings (defective in STP1 synthesis) indicate that STP1 accounts for the majority of glucose transport activity of *Arabidopsis* seedlings. Despite this the *Atstp1* mutant appears to grow and develop normally (Sherson *et al.,* 2000). This suggests that perhaps the *At*STP transporters have overlapping functions. A putative tobacco antisense transgenic line with inhibited hexose transporter activity exhibits a marked phenotype. Transgenic plants display stunted growth, decreased root system and an increased dry weight shoot/root ratio (unpublished data within Delrot *et al.,* 2000). It is postulated that this result highlights the requirement of hexose transport for normal plant growth and development. More analysis of this transgenic is required to be certain the antisense construct inhibits hexose transport specifically.

1.4 Regulation of the synthesis and activity of sugar transporters

Sugar transporters play an important role in the distribution of sugars between source and sink tissues. The uptake of sugars may be regulated by modulating the gene expression, protein translation or enzymatic activity of transporters.

Transcriptional regulation of sugar transporters results in specific developmental expression patterns (Wobus and Weber, 1999b; Ylstra *et al.*, 1998), modulation of expression during the sink-source transition of organs (Truernit and Sauer, 1995;

Riesmeier, 1993) and responses to abiotic factors (Truernit *et al.,* 1996; Noiraud *et al.,* 2000; Delrot *et al.,* 2000).

The diurnal regulation of the *Os*SUT1 and the *St*SUT1 sucrose transporters is mediated in a post-transcriptional manner (Hirose *et al.,* 1997; Kuhn *et al.,* 1997 respectively). Post-translational phosphorylation may also alter the activity of sugar transporters (Roblin, 1998).

In addition, H^+-sugar transporters may be regulated indirectly through H^+-ATPase activity, which generates the proton motive force required for H^+-symporter transport. In *Arabidopsis* 10 genes have been identified that encode H^+-ATPases (reviewed by Morsomme and Boutry, 2000). Several H^+-ATPases are expression in specific cell-types, such as, root hairs (Sussman, 1994), the phloem (DeWitt *et al.,* 1991) and developing seeds (Harper *et al.,* 1994). The synthesis and activity of H^+-ATPases may also be regulated by a variety of environmental factors. For example, the expression of H^+-ATPases in several species is induced by salt treatment (Niu *et al.*, 1996), darkness (Harms *et al.,* 1994), exogenous sugars (Mito *et al.,* 1996) and mechanical stimulation (Oufattole *et al.,* 2000).

There is increasing evidence to suggest that the regulation of nutrient transporter genes is sensitive to the concentration of transport substrate within the cell. For example, nitrate transporter (NRT2) activity is initially induced when N-starved roots are treated with NO_3^- (Zhuo *et al.,* 1999). Conversely, SO_4^-, inorganic phosphate and ammonium transporter activities are induced when the respective transport substrate is limiting (Clarkson *et al.,* 1990; Wang *et al.*, 2000). In addition, an *Arabidopsis* sucrose transporter gene is repressed by its transport substrate. Of the sugar treatments tested, only sucrose regulated the expression of this transporter (Chiou and Bush, 1998).

Moreover, the regulation of sugar transporter genes appears to be co-ordinated with that of genes related to carbohydrate translocation and partitioning (Gieger *et al.,* 1999). In addition, a number of genes encoding starch-mobilising enzymes including a putative sugar transporter are expressed at the same time during a diurnal light-dark cycle (Harmer *et al.,* 2000). The uptake of nutrients from the soil may also be co-ordinated. In sunflower roots, glucose uptake reportedly stimulates rubidium and water transport (Quintero *et al.,* 2000), although this may be quite indirect. Furthermore, the treatment of rutabaga plants with 1 mM glucose or sucrose resulted in an increase in both the gene expression of the AMT1 ammonium transporter, and ammonium uptake by the roots; similar results were seen in field pea seedlings (Kubik-Dobosz *et al.,* 2001)

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1.5 Regulation of gene expression by sugars

Sugars not only function as substrates for heterotrophic growth but also act as signalling molecules that can regulate genes associated with both source and sink metabolism. Such regulation may ultimately determine the pattern of carbohydrate production and allocation within the plant. Soluble sugar levels may influence carbon partitioning (Koch, 1996) shoot to root ratios (Wilson, 1988), induction of flowering (Corbesier *et al.,* 1998) tuber formation (Muller-Rober *et al.,* 1992) and senescence (Fujiki *et al.,* 2001).

Sugars modulate the expression of more than 40 genes (examples in table 1.2), including those involved in photosynthesis (Sheen, 1990; Krapp *et al.,* 1993, Smeekens and Rook, 1997; Pego *et al.,* 2000), glyoxylate cycle (Graham *et al.,* 1994), storage-related proteins (Martin *et al.,* 1997), nitrogen metabolism (Lam *et al.,* 1994), defense mechanisms (Heber *et al.,* 1996), senescence and cell wall modification (Fujiki *et al.,* 2001).

Table 1.2 Some sugar-responsive genes

GENE	PROTEIN	SUGAR EFFECT	SPECIES	REFERENCE
AGPase	ADP-glucose pyrophosphorylase	suc induction	Potato	Muller-Rober *et al.*, 1990
α-Amy	α-amylose	glc increase mRNA instability	Rice	Chan and Yu, 1998a/b
APL3	ADP-glucose pyrophosphorylase	suc induction	*Arabidopsis*	Rook *et al.*, 2001
ASN1	Asparagines synthetase	glc repression	*Arabidopsis*	Lam *et al.*, 1994
ATP-δ	δ-subunit of thylakoid ATPase	glc repression	*Chenopodium*	Krapp *et al.*, 1993
β-GLUC	β-glucosidase	glc repression	*Arabidopsis*	Fujiki *et al.*, 2000
CAB	Chlorophyll a/b binding protein	glc repression	*Arabidopsis*	Dijkwel *et al.*, 1997
CHS	Chalcone synthase	glc and suc induction	*Arabidopsis*	Sadka *et al.*, 1994
INV1	Apoplastic invertase	glc induction	*Chenopodium*	Roitsch *et al.*, 1995
LHA1	H^+-ATPase	repressed by metabolisable sugars	Potato	Mito *et al.*, 1996
RBCS	Rubisco small subunit	glc repression	*Arabidopsis*	Cheng *et al.*, 1992
MS	Malate synthase	glc repression	Cucumber	Graham *et al.*, 1994
NR	Nitrate reductase	glc induction	*Arabidopsis*	Cheng *et al.*, 1992
PAT(B33)	Class I patatin	suc induction	*Arabidopsis*	Martin *et al.*, 1997
PC	Plastocyanin	glc repression	*Arabidopsis*	Dijkwel *et al.*, 1996
PEPC1	C4 PEP carboxylase	sugar repression	Maize	Sheen, 1990
PR-genes	Pathogenesis-related genes	suc induction	*Arabidopsis*	Herbers *et al.*, 1996
SUC1	Sucrose transporter	suc repression (glc no effect)	*Arabidopsis*	Chiou and Bush, 1998
SUSY	Sucrose synthase	sugar induction	*Chenopodium*	Godt *et al.*, 1995
XET	Xyloglucan endoglycosylase	glc repression	*Arabidopsis*	Fujiki *et al.*, 2000

Generally, sugar depletion ('famine response') has been shown to enhance expression of genes involved in photosynthesis, reserve mobilisation and carbohydrate-export processes. Whereas abundant sugar resources ('feast response') induce those genes involved in storage and utilisation of carbohydrates (reviewed by Koch, 1996; Smeekens, 2000).

Investigation in to the mechanisms of the sugar signal transduction pathway is in its infancy. The use of sugar response mutants is proving useful in the identification of components within the sugar transduction pathway and interactions with other metabolic pathways (discussed in section 1.7). Yeast has been used as a model for sugar sensing in plants because Yeast controls metabolism and gene expression through characterised signal transduction pathways, which employing extracellular and intracellular sugar sensors (Gancedo, 1998; Rolland *et al.,* 2001).

1.6 Yeast; a sugar sensing model

In *Saccharomyces cerevisiae*, glucose is the favoured carbon source. In the presence of glucose, genes for fermentation and growth are induced, whereas genes associated with utilisation of alternative carbon sources are repressed. Yeast has developed sensing mechanisms to ensure co-ordination between supply of glucose from the environment to enzymatic machinery within the cell. This is accomplished by firstly, sensing the extracellular concentrations of sugars and regulating transport activity accordingly; and secondly by controlling metabolism within the cell by carbon catabolite repression (figure 1.3; reviewed by Ronne, 1995; Gancedo, 1998).

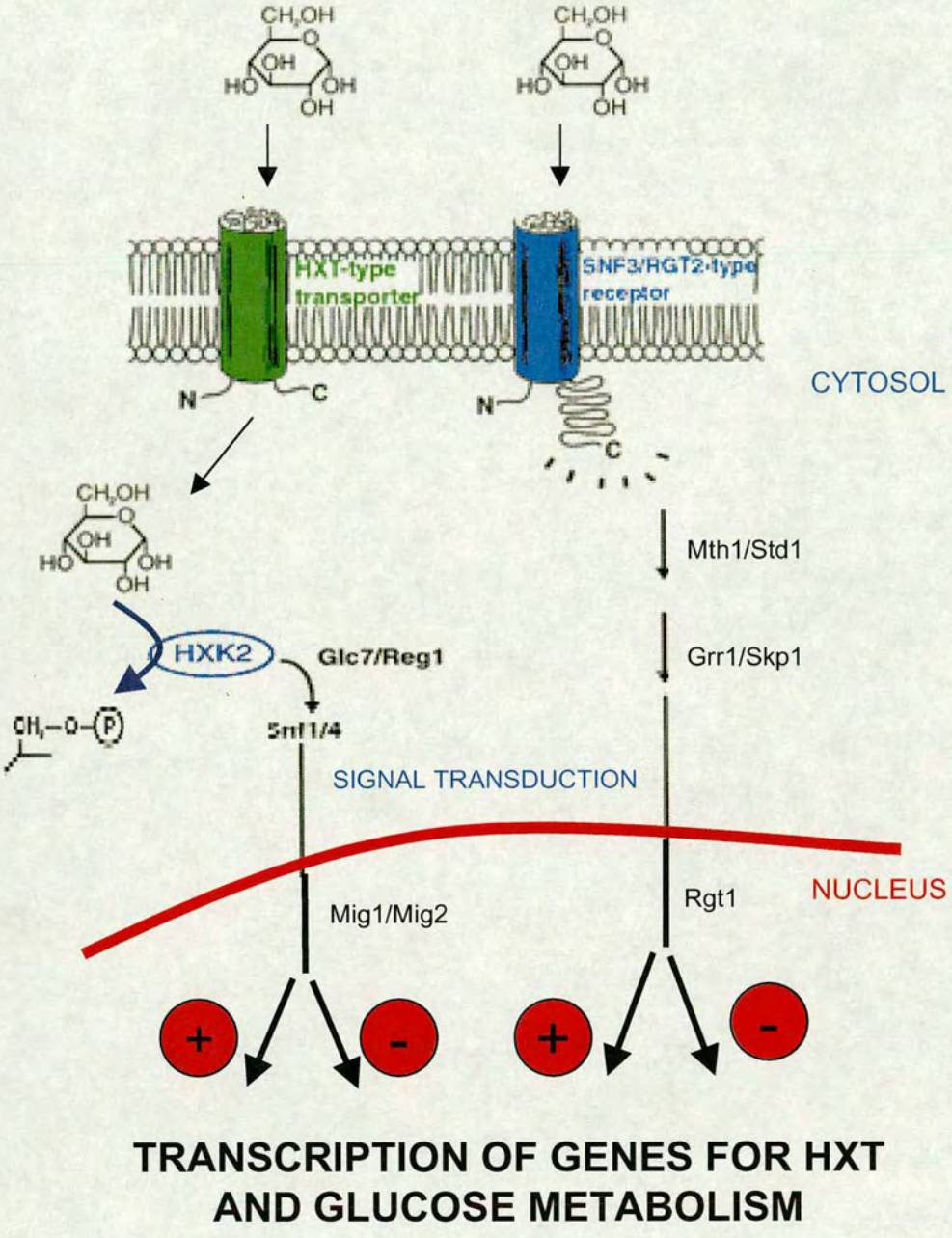


Figure 1.3 Glucose sensing in yeast.

Glucose sensors at the plasma membrane (blue tube) initiate a signal transduction pathway that can regulate gene expression independently of Hxk. Alternatively, glucose that is transported and phosphorylated by Hxk, which appears to transduce a signal via a number of protein kinases and regulatory proteins and ultimately modulate the gene transcription.

Extra-cellular sensing of glucose requires cell surface sugar sensors. Two hexose transporter homologues, Snf3 and Rgt2, with long cytoplasmic signalling domains and negligible transport activity have been identified, which may fulfil this role (Ozcan *et al.,* 1996). The concentration of glucose determines the nature and magnitude of gene regulation; for example, the Snf3 sensor responds to low levels of glucose by inducing the expression of high affinity glucose transporter genes, such as Hexose Transporter 2 (*HXK2*), *HXT6* and *HXT7*. The Rgt2 sensor responds to high levels of glucose and induces low affinity transporter genes, for example, *HXT1* (comprehensive review by Boles and Hollenberg, 1997).

Further investigation of the molecular mechanisms of glucose sensing and transport in Yeast has identified several regulatory proteins. The Grr1 regulatory protein is required to transduce the induction of *HXT1* to *HXT4* genes. Grr1 acts by regulating the DNA-binding protein Rgt1, which alleviates the Rgt1 transcriptional repression of the *HXT* genes (Johnston, 1999).

Furthermore, a two-hybrid screen revealed that Mth1 and Std1 regulatory proteins interact with the cytoplasmic signalling domains of the glucose sensors Snf3 and Rgt2. Therefore, it has been postulated that Mth1 and Std1 modulate the glucose regulation of the *HXT* genes by interacting and inhibiting the sensory function of the Snf3 and Rgt2 transporter homologues (Lafuente *et al.,* 2000; Schmidt *et al.,* 1999).

The glucose transport activity limits the provision of intracellular glucose and hence the amount of glucose available for downstream metabolism. In the Yeast cell, glucose can be phosphorylated by the kinase activity of either hexokinase (Hxk1 or Hxk2) or glucokinase (Glk1). Phosphorylated glucose is ultimately converted through the glycolytic pathway to ethanol (Herrero *et al.,* 1995).

An important feedback-mechanism that operates in Yeast is that of carbon catabolite repression, whereby the sensing of glucose results in the activation of glycolytic genes and the inhibition of those genes associated with gluconeogenesis (Gancedo, 1998). For carbon catabolite repression to occur the transport and phosphorylation of glucose is required (deWinde *et al.,* 1996). Hxk2 appears to mediate a long-term glucose repression of gluconeogenesis-related genes (Hofmann *et al.,* 1999). Key components of the catabolite signal transduction pathway are the Snf1 protein kinase and the Glc7 protein phosphatase type I. Snf1 appears to control transcriptional regulatory proteins, such as the repressors Mig1 and Mig2, by phosphorylation and inactivation (Hardie *et al.,* 1998; Gancedo, 1998)

The internal sensor for catabolite repression is still unclear. Initially, the requirement for Hxk catalytic activity implicated either a dual catalytic and regulatory function for Hxk2 (Ma *et al.,* 1989, Rose *et al.,* 1991) or a need for the accumulation of sugar-phosphates to trigger signalling (Rondez-Gil *et al.,* 1998). More recently, work with Yeast mutants has separated the catalytic activity of Hxk2 from glucose repression of gene expression, which suggests that Hxk2 may have a separate sensory domain that can transduce glucose signals without kinase activity (Mayordomo and Sanz, 2001; Hohmann *et al.,* 1999). A region of *HXK2* has also been identified that is important for nuclear targeting of the kinase, which raises the possibility of Hxk interacting, directly or indirectly, with DNA-regulatory proteins. Furthermore, this region of the *HXK2* gene is also required for the glucose repression of the *SUC2* invertase gene (Herrero *et al.,* 1998).

Alternatively, glucose phosphorylation by Hxk may result in the activation of cAMP synthesis and subsequent signalling that may transduce a glucose signal. The G-protein coupled receptor Gpr1, acts in concert with glucose phosphorylation under high glucose concentrations (Kraakman *et al.,* 1999). There is no direct evidence for

the binding of glucose to Gpr1 but it is required for the extracellular sensing of glucose, which implies that Gpr1 interacts with the Snf3 and Rgt2 glucose sensors (Rolland *et al.,* 2000). In addition, Gpr1 can activate a G protein (Gpa2), which in turn activates adenylate cyclase initiating a cAMP-signalling cascade. Cyclic AMP signalling can also result in carbon catabolite repression. These factors taken together raise the possibility that a cAMP signalling pathway may initiate glucose regulation of gene expression (Colombo *et al.,* 1998).

1.7 Sugar signal transduction pathway in plants

Sugar sensing in multi-cellular plants is likely to be more complex than that of unicellular Yeast cells, with the added interactions of source and sink organs for example. However, similar mechanism for sugar signalling may exist. The sugar signalling pathway in plants can modulate gene expression, post-translational processing, and protein activation. Using such sugar responsive genes as markers several sugar signal transduction pathways have been identified, e.g., hexose-specific and sucrose-specific regulatory pathways. A number of downstream components of these pathways have been isolated and putative extracellular and intracellular sugar sensors have been proposed.

Key components of the signal transduction pathway

The molecular mechanism employed in modulating gene expression can occur either through transcriptional or post-transcriptional processing. The repression of photosynthetic genes by carbohydrates occurs predominantly through transcriptional control (Sheen *et al.,* 1990). Post-transcriptional control can enhance, diminish or override that exerted at the transcriptional level. For example, the α-*Amy* gene

family demonstrates a sugar-repressive mechanism that operates via mRNA transcript instability (Chan and Yu, 1998a and 1998b).

Sugar-responsive transcriptional regulation is dependent on *cis*-elements located within the promoter region of the responsive genes. Several sugar responsive *cis*-elements have been identified in plant sugar-responsive genes. For example, the sugar responsive element (*SURE*) from the potato class I patatin protein (Grierson *et al.,* 1994) and similar elements have been identified in the sugar responsive promoters of rice, *Arabidopsis* and maize (Yu *et al.,* 1992; Shaw *et al.,* 1994; Fu *et al.,* 1995). Several unrelated sugar responsive *cis* elements have also been identified, such as, elements within the cucumber *MS* and *ICL* promoters (Graham *et al.,* 1994; De Bellis *et al.,* 1997). The identification of different sugar responsive *cis* elements suggests that a number of trans-acting transcription factors exist in plants. DNA binding proteins that may regulate sugar responsive genes include, storekeeper (Zourelidou *et al.,* 2002) and SPF1 (Ishigwo and Nakamura, 1994).

Sugars can also alter the protein expression and activity through translational and post-translational processing (Rook *et al.,* 1998). For example, high glucose concentrations can result in the inactivation of proteins and lipids by glycation (Baynes *et al.,* 1989; Bilan and Klip, 1990).

To date, only a few components of the sugar signal transduction pathway have been identified, such as SNF1-like protein kinases (Halford and Hardie, 1998). An experiment with transgenic lines harbouring antisense *SNF1* constructs demonstrated that sugar-regulated gene expression is altered in these plants (Purcell *et al.,* 1998). The *prl1* mutation results in transcriptional de-repression of glucose sensitive genes, defining a novel suppressor function in glucose signalling. The *PRL1* gene encodes a regulatory WD 40 protein that interacts with SNF1 in the

presence of sugar. It is postulated to function in plant sugar-regulated gene expression by acting as a negative regulator of SNF1 homologues; whereby the PRL1 protein binds to SNF1-like proteins, alleviating the SNF1 repression of gene transcription (Bhalereo *et al.,* 1999).

Furthermore, experimentation with pharmacological inhibitors has identified a range of protein phosphatases, Ser/Thr protein kinase and 14-3-3 proteins that are involved in the sugar regulation of gene expression (Otho *et al.,* 1995; Takeda *et al.,* 1994; Fujiki *et al.,* 2000, Cotelle *et al.,* 2000). The role of Ca^{2+} and calmodulin in sugar signalling also appears to be important (Vitrac *et al.,* 1999).

Experiments investigating the regulation of sugar-responsive genes upon treatment with various sugar analogues have revealed sugar-specific transduction pathways in plants. Three sugar signalling pathways have been proposed to date; sucrose-specific sensing, hexokinase-independent sensing and hexokinase-dependent sensing (figure 1.4).

Sucrose-specific signalling

The sucrose-specific signalling pathway was revealed by the identification of 2 genes, transcription factor ATB2 and a sucrose transporter; that were repression by sucrose alone (glucose and fructose had no effect) (Rook *et al.,* 1998; Chiou and Bush, 1998). Furthermore, the sucrose isomer palatinose can act as a signal in the regulation of sugar:starch ratios (Fernie *et al.,* 2001), plant development (Bornke *et al.,* 2002) and pathogen perception (Sinha *et al.,* 2002). As this isomer is not hydrolysed to glucose and fructose, these results are consistent with a sucrose-specific signalling pathway.

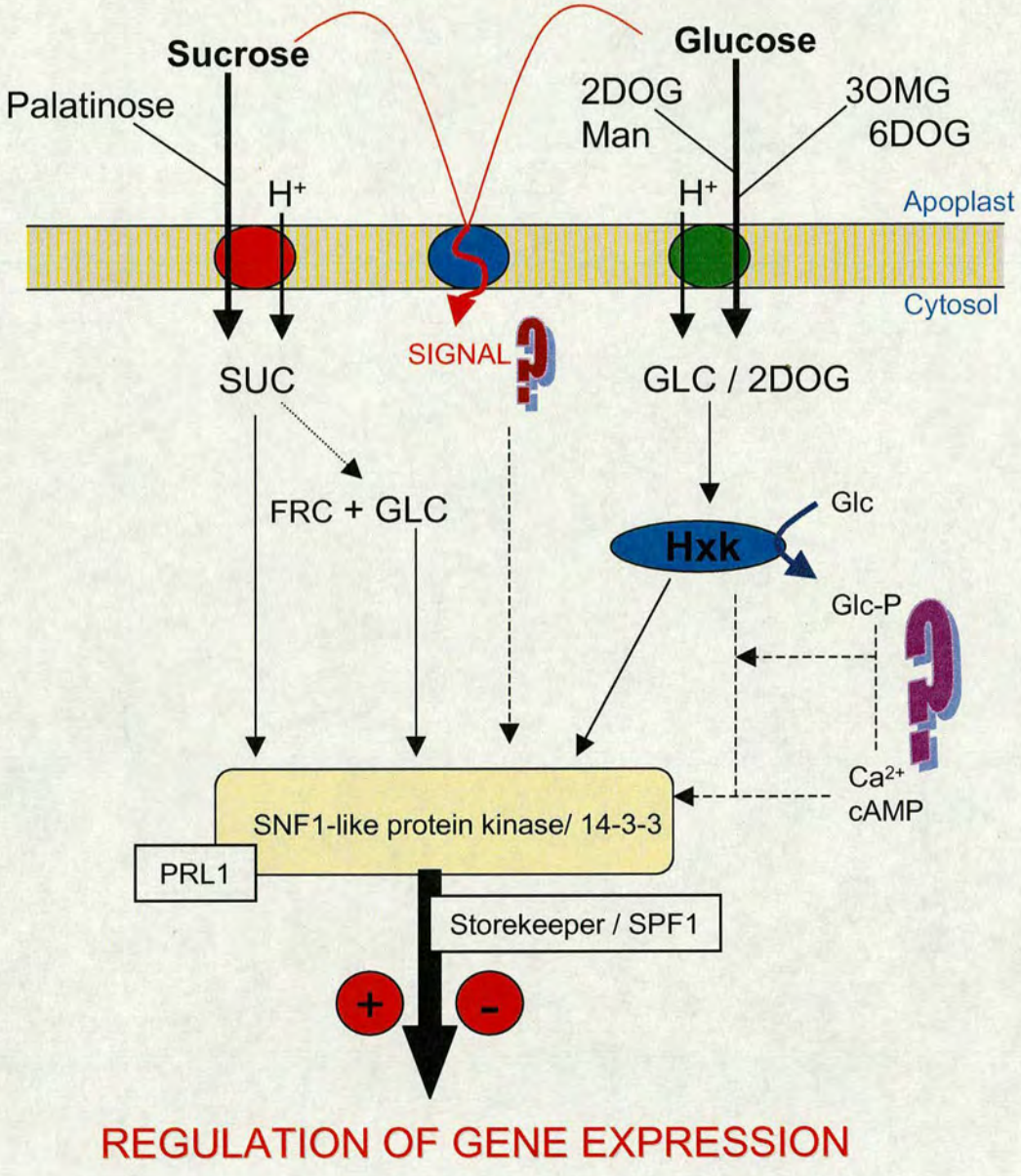


Figure 1.4 Diagrammatic representation of sugar-sensing in plants.

Sucrose symporters (red circle) provide intracellular sucrose, which can regulate gene expression specifically. Monosaccharide symporters (green circle) allow the uptake of hexose and analogs. Also a potential sugar sensor at the plasma membrane (blue circle) may trigger sugar-signalling. Glc, 6-DOG and 3OMG can initiate a Hxk-independent signal transduction pathway. Glc, Man and 2DOG are substrates of hexokinase; Hxk-dependent signalling may be initiated by Hxk activity, sugar-P, Ca^{2+} or cAMP. The signal transduction pathway includes protein kinases, regulatory proteins and transcription factors, which are required to induce or repress gene expression.

Hxk-independent sugar signalling

To elicit Hxk-independent sugar signalling, glucose (or analogue) acts as a signal without phosphoryation or further glycolytic metabolism occurring. Evidence for Hxk-independent signalling is based on experiments using hexose analogues that are not substrates for Hxk, such as 6-deoxyglucose (6-DOG) and 3-O-methyl glucose (3-OMG). In *Chenopodium* the expression of an invertase and sucrose synthase gene was induced by 6-DOG treatment (Roitsch *et al.,* 1995). In *Arabidopsis* roots the expression of the class I patatin gene was induced by 3-OMG (Martin *et al.,* 1997).

As previously described in Yeast, 2 transporter-like proteins (Snf3 and Rgt2) act as extracellular sugar sensors in the regulation of hexose transporter genes (*HXT*). A similar transporter-like protein Rco3 has been identified in *Neurospora crassa* that may act as a sensor (Madi *et al.,* 1997). Also, in mammals Glut1 and Glut2 transporter-like proteins are proposed to function as sensors (Bandyopadhyay *et al.,* 2000). There is mounting evidence to suggest transporter homologues may play an important role in plant sugar sensing; and could possibly have a sensory role in the sucrose-specific and hexokinase-independent signalling pathways (reviewed by Rolland *et al.,* 2001).

In *Arabidopsis* putative transporter-like proteins with long cytoplasmic tails have been identified but their function and possible role in sugar sensing has yet to be determined (Lalonde *et al.,* 1999). SUT2 is a sucrose transporter-like protein that shows negligible transport activity in Yeast (Barker *et al.,* 2000). In tomato, the *SUT2* mRNA is responsive to sucrose and co-localises with other sucrose transporters. Therefore, it is postulated SUT2 acts as a putative sugar sensor by regulating the transport activity of neighbouring transporters (Barker *et al.,* 2000).

Hxk-dependent sugar signalling

Hxk-dependent sugar signalling pathway requires the phosphorylation of glucose or analogues to transduce a signal. Therefore, experiments were conducted with the sugar analogue 2-deoxy-D-glucose (2-DOG), which is a substrate for Hxk but not thought to be further metabolised. In a number of experiments exogenous 2-DOG treatments resulted in the glucose repression of gene expression (Jang and Sheen, 1994; Graham *et al.,* 1994; Dai *et al.,* 1999). In experiments using transgenic *Arabidopsis* expressing the Yeast *HXK2* gene, an increased catalytic activity was observed, whereas glucose-regulated gene expression was less sensitive. It is postulated that these finding indicate a regulatory role for Hxk that is separable from the phosphorylation activity, like that seen in Yeast. However, the exact role of Hxk2 in sugar sensing remains controversial (Halford *et al.,* 1999).

1.8 Sugar-response mutants highlight interactions between signalling pathways

The aim of identifying components of the sugar-signalling pathway has lead to the isolation of mutants defective in their response(s) to sugar. Two main approaches for mutant screening in *Arabidopsis* have been employed (table 1.3); sugar response mutants have been isolated that are either defective in the expression of a specific sugar-regulated gene or develop normally when grown on sugar levels that inhibit wild-type growth.

Table 1.3 Screening conditions used in the isolation of sugar-response mutants.

MUTANT	SCREEN	PHENOTYPE	LOCUS/GENE PRODUCT	REF
rsr reduced sugar response	90 mM glucose	non-induction of pat::gus		Martin *et al.*, 1997
lba low-level β-amylase	175 mM sucrose	reduced induction of gene. *Landsberg erecta* ecotype is a naturally occurring lba.		Mita *et al.*, 1997a
hba high-level β-amylase	175 mM sucrose	constitutive expression independent of sugar.		Mita *et al.*, 1997b
cai carbohydrate insensitive	100 mM sucrose, low N	leaves develop with low anthocyanins and high chlorophyll		Boxall *et al.*, 1996
mig mannose-insensitive germination	7.5 mM mannose	no inhibition of germination (proposed defective Hxk2 signalling)	one of the mig's is a putative fructokinase mutant (frk2)	Pego *et al.*, 1999; Pego and Smeekens, 2000
gin glucose insensitive	330 mM glucose	normal of seedling development and true leaves	*gin1* is allelic to *aba2* *gin6* is allelic to *abi4*	Zhou *et al.*, 1998
sun sucrose uncoupled	88 mM sucrose	defective pc::luc repression	*sun6* is allelic to *abi4*	Dijkwel *et al.*, 1996
prl1 plieotropic regulatory locus1	175 mM sucrose or glucose	hypersensitivity arrested growth. affected by phytohormones.	PRL1 encodes a WD-40 protein	Nemeth *et al.*, 1998
sis sugar insensitive	300 mM sucrose or glucose	suc-insensitive seedling development,	*sis1* is allelic to *ctr1* *sis4* is allelic to *aba2* *sis5* is allelic to *abi4*	Gibson *et al.*, 1998
isi impaired sucrose induction		defective in suc-induced expression of AP2L gene	*isi3* is allelic to *abi4* *isi4* is allelic to *aba2*	Rook *et al.*, 2001

Characterisation of such mutants has revealed that many are defective in phytohormone response or metabolism; for example, the *prl1* mutant is hypersensitive to sugars as well as abscisic acid (ABA), ethylene, cytokinin and auxin (Nemeth *et al.,* 1998). Furthermore, a number of sugar-insensitive mutants, including *sis5* (Laby *et al.,* 2000), *sun6* (Huijser *et al.,* 2000) and *gin6* (Arenas-Huerto *et al.,* 2000) are allelic to the ABA insensitive mutant *abi4*. In addition, the *sis4* and *gin1* are mutants allelic to the ABA biosynthesis mutant *aba2* (Gibson, 2000). Experiments with the *gin1/aba2* mutant demonstrate that high sugar concentrations in the plant medium induce ABA synthesis, which is apparently required for a genetic response to high sugar levels to occur (Arenas-Huetero *et al.,* 2000). There appears to be a strong correlation between ABA and sugar signalling through seed development and the modulation of seedling carbon status (reviewed by Finkelstein and Gibson, 2001).

The glucose insensitive-ABA biosynthetic *gin1/aba2* mutant also appears it be defective in one branch of the ethylene signal transduction pathway (Zhou *et al.,* 1998). In addition, the sucrose insensitive *sis1* mutant is allelic to the ethylene response locus *ctr1* (Gibson *et al.,* 2001) and many ethylene response mutants have altered ABA responses. In fact, it is thought that a functional ETR1 (ethylene receptor) response pathway is required for a functional ABA signal transduction pathway to operate (Ghassemian *et al.,* 2000). The characterisation of sugar and hormone response mutants has identified at least a three-way interaction between ABA, ethylene and sugar signalling.

Moreover, interactions between sugar signalling and at least one other signal transduction pathway have been identified. For example, sugars may interact with light (Dijkwel *et al.,* 1997; Ciereszko *et al.,* 2001), cytokinins (Thomas *et al.,* 1997; Nemeth *et al.,* 1999), nitrogen (Lam *et al.,* 1998), phosphate (Nielsen *et al.,* 1998),

oxygen (Zeng *et al.,* 1998), auxin (Dewald *et al.,* 1994) or gibberellin (Perata *et al.,* 1997) signalling pathways. Interestingly, in *sun6/abi4* mutant both sucrose and light regulation was defective (Dijkwel *et al.,* 1997), which highlights the interaction of light and sugar with ABA signalling.

It is possible that interplay with other signalling pathways will be identified; such interactions are likely to result in a co-ordinated regulation of *Arabidopsis* development. However, perhaps more caution should be taken when interpreting such interactions. For example, the connections between sugar and the stress-inducible hormones, ABA and ethylene may only exist under the developmental stress conditions imposed by the mutant screens (Gazzarrini and McCourt, 2001). Alternatively, transgenic plants or mutants that contain raised levels of sugars relative to wild-type, may also be altered in osmolyte levels. Therefore, it is possible that interactions with stress signalling pathways may be caused by defective osmolyte transport between subcellular compartments or different organs; alternatively, osmolyte metabolism may impinge on sugar or redox signalling (Hare *et al.,* 1998).

Furthermore, the addition of exogenous sugars at non-physiologically high concentrations may cause deviations in normal metabolism, such as changes in AMP/ATP ratios, pH and calcium levels, which in turn may cause changes in multiple signal transduction pathways (Gibson and Graham, 1999). Elevated sugar levels in plants may result in increased glycation of proteins. This is the non-enzymatic glucosylation of proteins and lipids (Baynes *et al.,* 1989), which can result in subsequent inactivation of a glucose transporter (Bilan and Klip, 1990), glucokinase (Murata *et al.,* 1993), calcium pumps (Gonzalez-Flecha *et al.,* 1993) and calmodulin (Kowluru *et al.,* 1989).

1.9 Regulation of plant development and biological processes by light

1.9.1 The signalling function of light

In plants, light functions as a source of energy via photosynthesis, and also as a 'signal' in the regulation of growth and development (Kendrick and Kronenberg, 1994). The general model for light signalling consists of the initiation of a signal cascade by photoreceptors that modulate gene expression or cellular physiology in order to elicit a particular growth response (Chamovitz and Deng, 1996). More than 100 genes and 26 cellular pathways are regulated by light (examples in table 1.4; Fankhauser and Chory, 1997; Xu and Johnson, 2001).

Plants can monitor the intensity, quality, direction and duration of light and in turn modulate their development in order to optimise the acquisition of energy for photosynthesis and synchronise reproductive development. These processes are collectively called photomorphogenesis (for reviews see Whitelam and Devlin, 1998; Deng and Quail, 1999).

In higher plants three distinct classes of photoreceptors allow for the detection of light in the visible spectrum. These are the red/far-red photoreversible phytochromes (Fankhauser, 2001), the blue/UV-A-absorbing photoreceptors including cryptochromes (Cashmore *et al.,* 1999) and phototropins (Briggs and Christie, 2002), and UV-B photoreceptor (s) - as yet uncharacterised.

Table 1.4 The effect of light upon the expression of a variety of plant genes.

GENE	LIGHT TREATMENT	EFFECT ON EXPRESSION.	PHOTO-RECEPTOR	REFERENCE
TUB1	FR and R dependent	repression	phyA	Leu *et al.*, 1995
Athb-2	FR R	repression	phyA phy (not A/B)	Carabelli *et al.*, 1996
Lhcb1,2,3	VLFR LFR	induction	phyA phyB	Hamazato *et al.*, 1997
XTR7	VLFR	repression	phyA	Kuno and Furuya, 2000
CHS	B	initial induction	cry1 and 2	Jenkins *et al.*, 2001
CO	B	induction	cry2	Guo *et al.*, 1998
ASN1	dark	induction	Thought to be phyA	Lam *et al.*, 1994
CDPK2	dark	induction		Frattini *et al.*, 1999
βGluc	dark	induction		Fujiki *et al.*, 2000
LHA1	dark	induction		Mito *et al.*, 1996

1.9.2 The role of photoreceptors in light perception

Phytochromes

Phytochromes are dimers consisting of identical 125 kDa polypeptide monomers, each with a tetrapyrrole chromophore moiety (Quail *et al.,* 1994). The photosensory function of the phytochrome dimers is typically based on their capacity for reversible photoconversion between the Red-absorbing Pr form (λ_{max} = 666 nm) and the Far Red-absorbing Pfr (λ_{max} = 730 nm), when R and FR light is absorbed sequentially. Phytochrome is synthesised in the Pr form, which in most cases is biologically inactive. The Pfr form is considered to be biologically active. The spectral absorption ranges of the phytochrome R and FR-absorbing forms are different, but overlapping so under most light conditions both forms will exist. The photo-equilibrium of Pfr to Ptotal is dependent on wavelength, and is 80% in R (600 nm), 3% in FR (730 nm) and 40% in Blue (450 nm) light. Thus B (and UV-A) light is quite effective in the photo-transformation of Pr, making it difficult to distinguish between B light responses mediated by phytochrome or specific B light receptors, or a combination of the two (Batschauer, 1998).

In *Arabidopsis* five phytochrome genes (*PHYA-PHYE*) have been cloned and characterised (Sharrock and Quail, 1998; Clack *et al.,* 1994). Phytochrome A (phyA) has been identified as a type I or light labile apoprotein, which is abundant in dark-grown seedlings but rapidly degraded upon photoconversion to the Pfr form. Whereas, phyB-E are type II or light stable apoproteins, which are present in lower quantities and not degraded by the light (Kendrick and Kronenberg, 1994).

The localisation of these phytochromes is not clear. Studies in lower plants suggest phytochromes are associated with the plasma membrane, probably in a manner

involving as yet unidentified protein – protein interactions. However, computational analysis of higher plant phytochromes reveals no motifs, domains or secondary structure to facilitate insertion or association with the plasma membrane (Nagy *et al.,* 2000). Previous studies support the view that in higher plants phytochromes are soluble proteins localised to the cytosol (Quail *et al.,* 1995; Furuya and Schafer, 1996). However, more recent reports demonstrate light-regulated nuclear localisation of phyB (Sakamoto and Nagatani, 1996). GFP fusions with phyA and phyB revealed that tagged proteins were localised to the cytosol in the dark and subsequently accumulated in the nucleus under light treatment (Nagy and Schafer, 2000). Furthermore, evidence is accumulating that demonstrates R light dependent nuclear translocation of phyB is significantly slower than phyA, which may have diverse effects upon the photo-regulation of gene expression by specific phytochromes (Yamaguchi *et al.,* 1999). For example, phyB interacts with transcription factors in the nucleus (Ni *et al.,* 1999) and thereby functions in the assembly or activation of complexes that bind the light responsive *cis* elements of various target genes (Martinez-Garcia *et al.,* 2000). The localisation of various phytochrome proteins to different cellular compartments could explain differences in their modes of action (Batschauer, 1998).

Phytochromes mediate a variety of light responses and the presence of multiple phytochromes is indicating of each phy having a distinct function. The availability of *Arabidopsis* mutants defective in one or more phytochrome has been useful in dissecting function. *Arabidopsis* mutants deficient in phyA (*phya)* were isolated due to the insensitivity of the seedlings to prolonged FR light. For example, wild-type FR light responses associated with the inhibition of hypocotyls elongation, promotion of cotyledon expansion, anthocyanin synthesis and the regulation of specific genes were not apparent in the mutant (Whitelam *et al.,* 1993; Johnson *et al.,* 1994; Barnes, *et al.,* 1996). The highly specialised phyA is responsible for initiating both

the Very Low Fluence Rate response and the High Irradiance Response following the exposure of plantlets to low or high FR irradiance levels respectively (Furuya and Schafer, 1996).

Etiolated *phyb* mutants display reduced sensitivity to the R inhibition of hypocotyl elongation and cotyledon expansion, but display wild-type responses to prolonged FR or B light (Koornfeef *et al.,* 1980; Reed *et al.,* 1993). The classical R/FR reversible induction, the low fluence response (LFR) and the continuous red light response (cR) are controlled by phyB and possibly other phytochromes (Furuya and Schafer, 1996). Furthermore, phyB has also been implicated in the light-promotion of *Arabidopsis* seed germination (Shinomura *et al.,* 1996) and in initiating shade avoidance responses via FR perception (Ballare, 1999).

It is evident, through analysis of phyA and phyB functions that there is considerable overlap in the perception and subsequent signalling in the presence of R or FR light. Furthermore, analysis of the *phya phyb* digenic mutant has highlighted functional redundancy between the phytochrome responses. For example, the R light mediated induction of *CAB* gene expression in both the *phya* and *phyb* monogenic mutants is comparable to the wild type response, although the *CAB* gene expression is severely reduced in the double mutant (Reed *et al.,* 1994). In addition, several R and FR light effects of the *phya phyb* mutant respond with similar sensitivity to wild type, for example, *Athb-2* transcript accumulation (Carbabelli *et al.,* 1996). This implies that other phytochromes or photoreceptors may play a role the mediation of such light responses, as well as phyA and phyB.

No mutants have been characterised for the remaining phytochromes (phyC-phyE) so distinct functions have not been identified. However, some Ws ecotypes appear to be *phyd* mutants. Initial analysis of light responses in these plants suggests that

phyD is responsible for some, but not all of the low R/FR ratio responses seen in the *phya phyb* mutant (Whitelam and Devlin, 1998).

UV-A/blue light photoreceptors

The *HY4* gene encodes the blue light absorbing photoreceptor, cryptochrome1 (cry1) (Ahmad and Cashmore, 1993). Characterisation of light grown *hy4* mutant seedlings revealed decreased cotyledon expansion, increased petiole elongation, flower stem elongation and leaf expansion, decreased anthocyanin accumulation and a reduced B light-induction of *CHS* gene expression (Jackson and Jenkins, 1995) The blue light induction of the *CHS* gene is independent of phyA and phyB (Batschauer *et al.,* 1996). The cryptochrome proteins are similar in structure to that of bacterial photolyases, but cry's have additional C-terminal extensions and no photolyase activity. The topology suggests two catalytically active chromophores are present, FADH and either a pterin or deazoflavin (Malhotra *et al.,* 1995). The over-expression of cry1 indicates the protein is cytosolic in the light (Lin *et al.,* 1996) and in a cry1-GFP transgenic line the tagged protein is located in the nucleus in the dark (Cashmore, 1998). The *CRY2* gene has also been cloned; analysis of the protein structure reveals a different C-terminal extension to that of cry1 (Lin *et al.,* 1998). Initial reports of the localisation of cry2 using GFP and GUS tagged proteins reveals constitutive expression in the nucleus (Kleiner *et al.,* 1999; Guo *et al.,* 1998).

CRY2 is allelic to the late flowering mutant (*fha1*) and is affected in photoperiodicity. Furthermore, cry2 positively regulates CONSTANS (CO), a transcription factor required for the long day promotion of flowering in *Arabidopsis* (Guo *et al.,* 1998). The cry1 and cry2 photoreceptors also mediate de-etiolation (Ahmed and Cashmore, 1993).

The phototropin UV-A/blue-light photoreceptor was originally identified by physiological and biochemical data that showed a strong correlation between phototropism and the blue-light mediated phosphorylation of a 120 kDa protein associated with the plasma membrane (Short and Briggs, 1994). The isolation of an *Arabidopsis* mutant lacking phototropism (*nph1*) and the 120 kDa protein (nph1) was further evidence that this protein may be a blue light photoreceptor (Liscum and Briggs, 1995). More recent characterisation of the non-phototropic hypocotyl locus (*NPH1*) has revealed that *NPH1* encodes a serine/ threonine kinase with 2 N-terminal domains characteristic of proteins that mediate light, oxygen and voltage-dependent responses (LOV domains). These LOV domains bind a flavin chromophore (FMN) (Cristie *et al.*, 1998; Christie *et al.,* 1999).

Further data demonstrates that nph1 is a blue light photoreceptor (comprehensively reviewed by Briggs and Cristie, 2002), which has been re-named phototropin (phot1). In fact, 2 phototropin photoreceptors are present in *Arabidopsis*, namely phot1 (nph1) and phot2 (nph2) (Briggs and Cristie, 2002). Phototropins mediate the blue light regulation of phototropism, chloroplast relocation and stomatal opening (Huala *et al.,* 1997; Sakai *et al.,* 2001; Jarillo *et al.,* 2001; Kinoshita *et al.,* 2002).

UV-B photoreceptors

A well-studied UV-B light response is the accumulation of flavonoid pigments in plants, which are thought to act in UV-shielding (Beggs and Wellmann, 1985; Christie and Jenkins, 1996). Phytochromes also absorb UV-B light, but the inability of FR light to reverse the UV-B response, which suggests that there is a distinct photoreceptor responsible for transducing UV-B light perception (Batschauer, 1998; Beggs *et al.,* 1995). To date, no such photoreceptor has been characterised at the molecular level.

1.9.3 Transduction pathway and light responsive genes

The genetic approach used to study the light signal transduction components has lead to the identification of many *Arabidopsis* mutants impaired in normal light regulation. The developmental program that plants follow in the light is referred to as photomorphogenesis and affects many aspects of plant growth. For example, stem elongation, leaf and chloroplast development and transcript accumulation of light regulated genes. Mutants that maintain a photomorphogenic phenotype in the dark (fail to exhibit an etiolated phenotype) have been isolated, e.g., *det* (de-etiolated), *cop* (constitutive photomorphogenesis) and *lip* (light independent photomorphogenesis) mutants (Chory *et al.,* 1989, Deng *et al.,* 1992; Frances and Thompson, 1997).

Etiolation or skotomorphogenesis is the developmental programme followed by plants grown in complete darkness. The repression of genes related to photomorphogenesis may be responsible for etiolation (Chory *et al.,* 1989). Mutants that show attributes of etiolation when maintained in light have also been isolated, e.g., *hy* (long hypocotyls of light-grown plants) mutants (Koornneef *et al.,* 1980).

The cloning and characterisation of the mutant loci of such mutants has identified protein kinases and transcription factors involved in the light signalling transduction pathway (reviewed by Schwechheimer and Deng, 2001). For example, the *COP1* gene encodes a transcription factor that acts as a repressor of photomorphogenesis by physically interacting with the transcriptional activator HY5 (Ang *et al.,* 1998)

Biochemical evidence suggests GTP-binding proteins, Ca^{2+}, calmodulin and cGMP function in mediating phytochrome-controlled signalling (Bolwer *et al.,* 1994). The increasing data from light-response mutants, genetic analysis and biochemical

experiment has established many intermediates of the light signalling pathway (for reviews see Barnes *et al.,* 1997; Kuno and Furuya, 2000)

1.10 Diurnal and circadian regulation of gene expression in plants

Plants grown in a 'normal' 24 h light-dark cycle restrict a range of processes to specific times during the diurnal cycle. Through this mechanism plants may optimise the expression of genes involved with specific biological and metabolic processes to the most favourable times of the day, for example, genes associated with photosynthesis are expression in the light. It appears that in addition to simply responding to daily environmental cues, such as the light-dark transition, plants have evolved an endogenous oscillator, or clock mechanism to control the timing of gene expression through the diurnal cycle. The regulation of cellular and physiological activity by such clocks is revealed under constant conditions. Processes that persist in an absence of environmental cues, with a rhythmic periodicity of approximately 24 h, are likely to be controlled by a circadian clock (Edmunds, 1988).

Many facets of plant development and cellular processes have circadian rhythm, such as the expression of genes associated with photosynthesis and related biochemical processes (Harmer *et al.,* 2000; Krep, 2000; Schaffer *et al.,* 2001), cytosolic concentration levels of free Ca^{2+} (Wood *et al.,* 2001) protein phosphorylation (Nimmo, 2000), chloroplast movement (Johnson, 1998), hypocotyl elongation (Dowson-Day and Millar, 1999), cotyledon and leave movement (Johnson, 1998) and the timing of flowering (Reeves and Coupland, 2000).

The circadian clock allows plants to 'anticipate' the daily changes in the environment, such as the timing of dusk and dawn. By this process, plants can

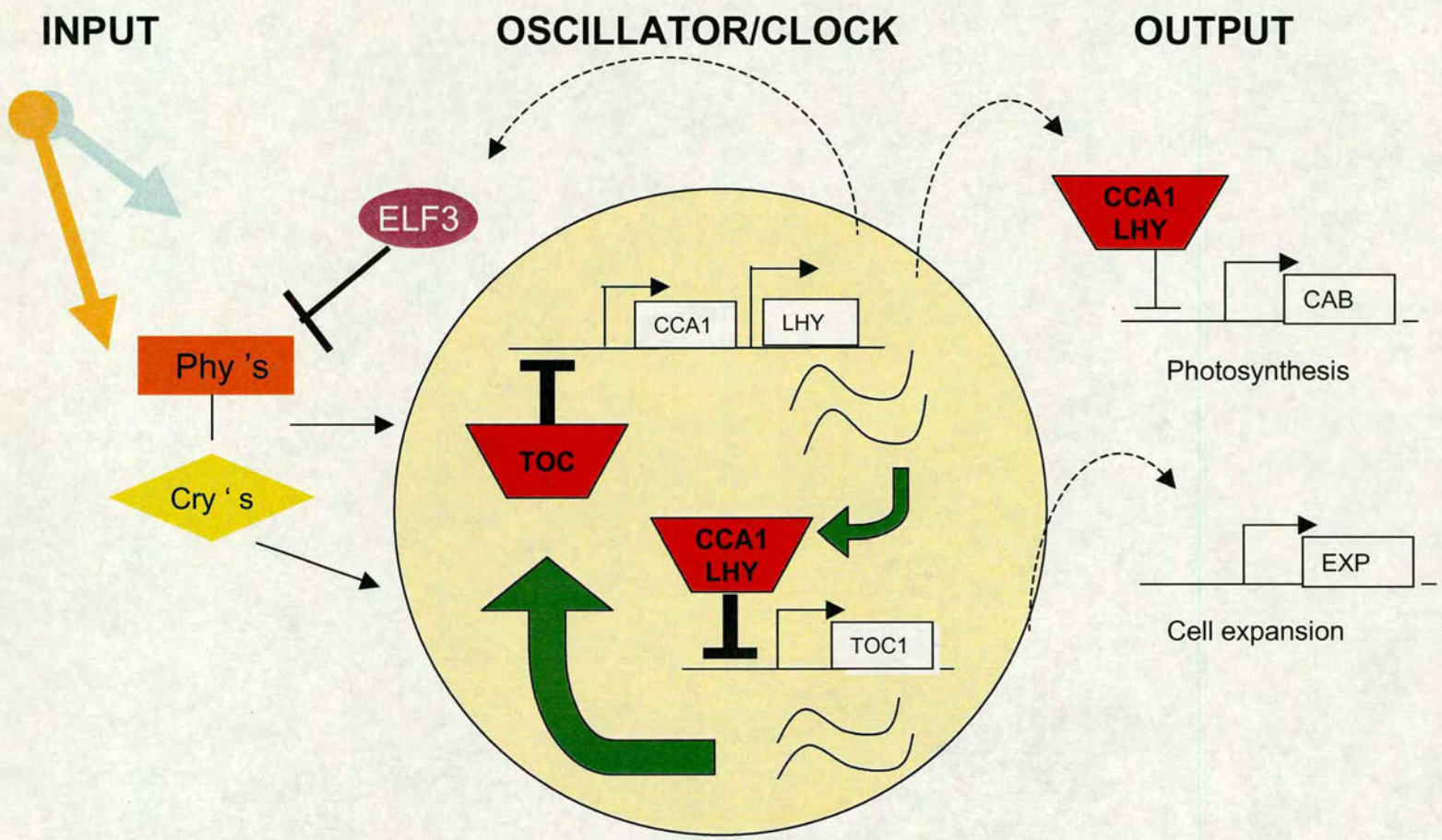


Figure 1.5 The proposed mechanism of the circadian clock in *Arabidopsis.*

Light input via photoreceptors (Phys and Crys) entrains the circadian clock to environmental conditions, this input may be modulated by ELF3. The clock/oscillator consists of an autoregulatory feedback loop of CCA1, LHY and TOC1 expression. The clock generates signals that are transduced via the output pathway to produce overt circadian rhythms; regulating photosynthesis (CAB) and cell expansion (EXPansins).

detect seasonal changes in day-length or photoperiod and may adjust cellular and physiological processes accordingly, such as reproductive activity and seed germination (Carre, 2001; Suarez-Lopez *et al.,* 2001).

The classic conceptual model of the circadian clock comprises of input pathways, a self-sustaining oscillator, and output pathways that are connected to clock-regulated processes (figure 1.5). The input pathways transduce time-keeping signals from environmental cues, such as the diurnal light-dark transitions. Such cues entrain or reset the period and phase of the oscillator to match the solar cycle. In *Arabidopsis* phyA, phyB, phyD, phyE, cry1 and cry2 photoreceptors have been implicated in clock entrainment (reviewed by Devlin and Kay, 2001).

However, a quadruple mutant lacking phyA, phyB, cry1 and cry2 retains a robust circadian rhythmicity, indicating that those photoreceptors are not essential components of the oscillator (Yanovsky *et al.,* 2000). Furthermore, the ability of plants to respond to light-input signals may vary depending on the time of day; this phenomenon is known as gating. The gating of light input is mediated in part through the transcriptional regulation of photoreceptors (Neff *et al.,* 2000) and by rhythmic activation or inhibition of signalling molecules. It is proposed that EARLY FLOWERING 3 (ELF3) is responsible for the perception of light by the clock, and therefore may play a role in gating (reviewed by Carre, 2002). In *Arabidopsis*, the ELF3 nuclear protein is expressed rhythmically, peaking in the middle of the subjective night (Hicks *et al.,* 2001), and interacting with the phyB photoreceptor (Liu *et al.,* 2001).

Non-photic signals may also entrain circadian rhythms in plants. For example, changes in temperature (Johnson, 1998) or imbibition of seeds (Zhong *et al.,* 1998).

In recent years, a number of components of the circadian clock have been identified; although the molecular basis of the circadian oscillator remains unknown. However, broad outlines of the working of the Arabidopsis clock have been proposed, whereby a negative feedback loop in the regulation of *TOC1*, *CCA1* and *LHY* gene expression constitutes the central oscillator mechanism (for reviews see Barak *et al.,* 2002 and Yanovsky and Kay, 2001; Mizuno *et al.,* 2002).

The output pathways provide a link between the oscillator and the circadian rhythms of a variety of genes in *Arabidopsis*, such as *CAB* (Millar and Kay, 1996), *NAI2* (nitrate reductase; Pilgrim *et al.,* 1993), *CAT* (catalase, McClung, 1997), *CCR2* (cold-circadian rhythm RNA binding2; Kreps and Simon, 1997), *β-Amy* (Chandler *et al.,* 2001). It has been postulated that the 'clock' has distinct output pathways that allow for the transcription of genes at different times during the subjective diurnal cycle. This is consistent with the differential expression of a number of clock-controlled genes, for example, *CAB* is expressed early in the subjective day (Millar and Kay, 1996), whereas *CAT3* is expressed in the late afternoon (Zhong and McClung, 1996) and *CCR2* at the beginning of the subjective night (Carpenter *et al.,* 1994).

More recently genome-wide studies have revealed the co-ordinated expression of functionally associated-genes through the subjective diurnal cycle. Harmer *et al.,* (2000) revealed the clustering of genes associated with cell elongation and cell wall synthesis at subjective dusk and during the subjective night respectively.

1.11 Dark-inducible gene expression

In plants grown in a diurnal cycle, a number of biological processes are restricted to the dark phase. A number of genes in *Arabidopsis* have been classified as dark-

inducible, such as genes related to starch mobilisation (Geiger *et al.,* 2000), amino acid biosynthesis (Lam *et al.,* 1994), H^+-ATPase activity (Harms *et al.,* 1994), and cell wall elongation (Nicol and Hofte, 1998). A second category of dark-inducible genes are expressed in plants exposed to prolonged darkness (greater than 24 h), including genes associated with stress or adaptive responses, such as senescence (Scheumann *et al.,* 1999), and pathogen attack (Sessa *et al.,* 1995).

In addition, the expression of the monosaccharide transporter *AtSTP1* gene is induced in dark-incubated seedlings (Hemmann, 2000). The substrate specificity of *At*STP1 is similar to the sugar composition of the primary cell wall, which argues for a possible role in the salvage of sugars liberated from the cell wall during turnover and modification (Sherson *et al.,* 2000). Therefore, the observations below may have relevance to the potential function(s) of *At*STP1.

Cell expansion

In plants, the cell wall is the major carbohydrate sink comprising 20-50% dry weight of the plant and 70-80% of total carbohydrate (Pennig de vries *et al.,* 1974). Cellulose and callose synthases produce cellulose and callose respectively, which are the only polysaccharides known to be made at the plasma membrane (Delmer and Amor, 1995). Other interlocking glycans and pectins are synthesised and modified in the golgi apparatus, then packaged into secretory vesicles and transported to the cell wall. Further modification of cell wall components occurs *in situ* at the wall, such as de-esterifcation of pectins, phenolic crosslinking and hydrolysis of glucans (McCann *et al.,* 1995). Such steps are thought to continual strengthen the xyloglucan/pectin matrix that binds and maintains the physical integrity of the cellulose network (Cosgrove, 2000). The mode of cell expansion is thought to require the hydrolysis, transfer or movement (slippage) of xyloglucan

polymers before the cellulose network can be extended (Nishitami, 1992; Rose and Bennett, 1999). Cell wall loosening agents, such as expansins, xyloglucan endoglycosylases (XET) and polysaccharide endo- and exohydrolases mediate such chemical modifications of the cell wall polymers (reviewed by Cosgrove, 1999).

The constant rearrangement incorporation of new polymers that results in cell expansion is proposed to be pH dependent. The acid growth theory refers to enhanced expansion of the cell wall at acidic pH (McQueen-Mason, 1997). Whereby the activation of H^+-ATPases, perhaps by auxin or other factors, lowers the pH of the cell wall and in turn stimulates the highly pH-dependent activity of expansins, XET, endo-glucanase (EGase) and glucohydrolase or exohydrolase (reviewed by Nicol and Hofte, 1998). According to the theory, acidification of the apoplast also induces K^+ uptake via hyperpolarisation of the plasma membrane (Claussen *et al.,* 1997). K^+ uptake promotes osmotic changes, which favour water influx across the plasma membrane, which as a consequence promotes cell expansion (Maurel, 1997).

Many of the genes associated with the cell elongation are dark inducible. For example, genes encoding an XET (Xu *et al.,* 1995), a Ca^{2+}-binding protein (Braam *et al.,* 1997), the LHA1 H^+-ATPase (Mito *et al.,* 1996), an oligoglucoside cleaving EGase (Nicol and Hofte, 1998) and a glucose cleaving β-D-glucan glucohydrolase (Roulin *et al.,* 2002). In addition, the expression of several genes associated with cell wall modification and expansion are classified as *TOUCH* (*TCH*) genes, which are induced in response to mechanical stimulation (reviewed by Braam *et al.,* 1997). Growth hormones such as, auxin, ethylene and brassinosteroids can also regulate cell wall loosening agents (Brummell *et al.,* 1994; Clouse, 2001). Therefore, it seems likely that the production and regulation of the cell wall is co-ordinated with other biological processes or events by a variety of signalling pathways.

As plant cell walls extend they change the size and shape of the cell, which can influence neighbouring cells, for example, interaction between pollen and stigma. Several classes of proteins physically link the plasma membrane to the carbohydrate matrix of the cell wall, and therefore have the potential to directly signal cellular events through their cytoplasmic kinase domain. For example, cell wall-associated kinases (WAKS) and cellulose synthase (reviewed by Anderson *et al.,* 2001).

Senescence

The exposure of plants to darkness results in the rapid depletion of the carbohydrate reserves in the leaves (Brouquisse *et al.,* 1998), and after prolonged darkness lipids and proteins derived from cell components contribute to respiration (Smart, 1994). In addition, carbohydrate starvation induces the catabolism of fatty acids and amino acids. The relationship between sugar starvation and dark-induced gene expression has not been greatly investigated. However, a set of dark-inducible genes are expressed in senescing leaves and may be repressed by sugars, e.g., β glucosidase, asparagine synthetase, phosphomannose isomerase and glyoxylate cycle genes (Fujiki *et al.,* 2001; Graham *et al.,* 1994).

Bleecker (1998) suggests senescence syndrome represents a salvage system that allows the retention of nutrients by the plant, rather than a process of programmed cell death to rid the plant of old and diseased tissue. This view is supported by the fact that during senescence the structural integrity of chloroplasts diminishes, thylakoid membranes breakdown, and the number of cytoplasmic ribosomes decrease, resulting in the loss cellular carbon and nitrogen; but the integrity of the plasma membrane, mitochondria and nucleus is maintained until the final stages of senescence (Nooden and Guiamet, 1997). Senescence-associated genes (SAGs)

include proteases, nucleases, lipid-, carbo-, nitrogen- metabolising enzymes and nutrient transporters, which are highly expressed during senescence (reviewed by Quirino *et al.,* 2000).

1.12 This Project

At the outset of this project the aim was to characterise the expression and regulation of the *STP1* transporter gene, with regard to exogenous sugar. To generate an *STP1*-driven luciferase reporter construct, potentially for use in a screening programme to isolate mutants impaired in *STP1* gene response to sugar. However, during the course of investigating the *STP1* sugar response, it became evident that the *STP1* gene was regulated by a number of biotic and abiotic factors. Therefore, the majority of this report is dedicated to the characterisation of such regulatory factors and the responses they elicit upon the synthesis and activity of the STP1 transporter, under a range of growth conditions and treatments.

The expression of the *STP1* gene is modulated by mechanical stimulation. This discovery occurred somewhat serendipitously, due to the manner in which experiments were performed. Therefore, a certain amount of variation between results is apparent, whereby the effect of mechano-stimulation is evident but was not understood at the time.

The following chapters describe the effect of various regulatory factors upon *STP1* gene expression and transport activity in plantlets grown either under condition light (chapter 3) or following entrainment to a diurnal growth regime (chapter 4). Due to the way in which the chapters are divided, a number of experiments are not displayed in chronological order. Hence, on occasion, they may not naturally lead one from another.

CHAPTER TWO: Materials and Methods

2.1 Biological Materials and chemicals

2.1.1 Plant material, growth and treatments

The wild-type *Arabidopsis thaliana* ecotype used was Columbia 0 (NASC accession N1092), unless otherwise stated. The transgenic and mutant strains used are described below:

stp1-1	STP1 knock-out mutant (Ws)	Sherson *et al.,* (2000)
sex4	starch excess mutant (Col0)	Zeeman *et al.,* (1998)
pgm1	phosphoglucomutase null mutant	Caspar *et al.,* (1985)
det3	de-etiolated mutant (Col)	Dr M. Campbell, University of Oxford, UK
aba2-1	abscisic acid deficient mutant (Col)	Dr S. Smeekens, University of Utrecht, Netherlands
abi4-1	abscisic acid insensitive mutant (Col)	Dr S. Smeekens
phyA	phyA-201 null mutant	NASC N6219
phyB	phyB-5 null mutant	NASC N6213
phyA/B	phyA/B null mutant	NASC N6224
elf3	early flowering mutant3	Dr A. Millar, University of Warwick, UK

Arabidopsis seeds were sterilised with 30% domestic bleach for 10 min, prior their distribution in a single row in the middle of the plant medium plates. Unless otherwise stated, the plates contained ½ strength Murashige and Skoog (1962) medium, 0.5% (w/v) MES, 0.8% (w/v) agar, pH adjusted to pH 5.7 with NaOH. The seeds on plates were incubated at 4°C for several days prior to their transfer to a particular growth regime. Plates were arranged vertically under the light, so plantlets grew along the surface of the plant medium. Unless otherwise stated plantlets were grown under continuous white light (~50 μmol m^{-2} s^{-1}) in a growth chamber.

The treatment of plantlets with water or various sugar solutions was conducted by pouring approximately 10 ml of solution to horizontally arranged plates of plantlets. The water or sugar solution was swirled around the plates so all plantlets were exposed to the treatment solution. These plates were subsequently incubated in either the light or the dark for an allotted time period. Following the respective treatments, the plates were drained of the treatment solution, and the treated-plantlets were blotted to remove excess fluid. The treated-plantlets were then harvested either whole or following the separation of the shoot and root tissues with a sharp scalpel. The harvested tissue samples were frozen in liquid nitrogen and stored at -70°C.

2.1.2 Bacterial strains and media

Escherichia coli strain DH5α; *Sup*E44 Δ*lac*U169 (Φ80*lac*ZΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1 (Hanahan, 1983).

Escherichia coli Top10; F-mcrAD (mrr-hsdRMSmcrBC) f80lac ZDM15D lac X74 deoR recA1 araD139 D(ara-leu) 7697 galU galK rpsL(Strr) end A1 nupG (Invitrogen, Paisley, UK).

Agrobacterium tumefaciens, strain GV3101 containing the Ti plasmid pMP90RK (Koncz and Schell, 1986; Van Larabeke *et al.,* 1974)

Bacteria were grown and maintained on Luria Bertani (LB; 10% (w/v) bacto-tryptone (Difco laboratories, Detroit, MI, USA), 5% (w/v) bacto-Yeast (Difco laboratories, Detroit, MI, USA), 10% (w/v) NaCl, at pH 7.0). Antibiotics were added to the LB agar for the selection of transformed bacteria (Sambrook *et al.*, 1989).

2.1.3 Plasmids

Vector	Source or Ref	Use
pBluescript II SK+	Altingmees and Short, 1989	Cloning of PCR products and sequencing
pGEM T-easy	Promega, Southampton, UK,	Cloning of PCR products and sequencing
pSP-*luc*+	Promega, Southampton, UK,	Generation of a luciferase gene reporter construct
pGreen 0179	Hellens *et al*., 2000	Ti binary vector
pBluescript-*CAB*	A. Millar, University of Warwick, UK	Amplification of *CAB* gene for a hybridisation probe
pBluescript-*STP1*	N. Sauer, University of Erlangen, Germany	Amplification of the *STP1* gene for the construction of reporter gene and a hybridisation probe

2.1.4 Chemicals

Unless otherwise stated all chemicals were supplied by Sigma Chemical Co. (Poole, Dorset, UK).

2.2 RNA isolation and analysis

2.2.1 Extraction of total RNA from plant tissue

The total RNA from ~100 μg of plant tissue was extracted using an RNeasy plant mini kit (Qiagen Ltd, Crawley, West Sussex, UK). The kit allowed the simultaneous processing of up to 12 tissue samples in 40 min. The protocol used was as stated in the manufacturer's handbook.

2.2.2 Quantification of RNA

RNA was quantified by measuring the A_{260}. An A_{260} value of 1 corresponds to approximately 40 μg ml^{-1} of RNA.

2.2.3 Horizontal gel electrophoresis

RNA samples were prepared for gel fractionation by drying aliquots under a constant vacuum. Upon complete desiccation samples were dissolved in 5 μl of RNA gel loading buffer. The loading buffer consisted of 1 X MOPS buffer (20 mM 3-[N-morpholino]-propansulphonic acid, 5 mM sodium acetate pH 7.0, 1 mM EDTA pH8.0), 50% (v/v) formamide, 6.6% (v/v) formaldehyde, 3% (w/v) ficoll type 400, 0.02% (w/v) bromophenol blue and 50 μg ml^{-1} ethidium bromide. Samples were heated to 60°C for 15 min prior to loading.

RNA samples were fractionated by electrophoresis through a 1.2% (w/v) agarose gel containing 6.6% (v/v) formaldehyde and 1 X MOPS buffer. Ribosomal RNA was visualised by UV trans-illumination. Equal loading of total RNA was verified by the qualitative comparison of the 18 S rRNA bands of each sample.

2.2.4 Northern Blotting

After fractionation, the RNA was transferred from the gel to Hybond N membrane (Amersham plc, Little Chalfont, Bucks, UK) by capillary action as described by Southern (Southern, 1975). A salt gradient from a reservoir of 10 X SSPE (1.8 M NaCl, 100 mM $NaH_2PO_4.H_2O$, 20 mM EDTA, pH 8.0) facilitated the transfer of RNA to the membrane. The RNA gel-blots were subsequently washed with 2 X SSPE. RNA was cross-linked to the membranes by UV irradiation at 0.4 Jcm^{-2}.

2.2.5 Radio-labelling of dsDNA probe by random priming

The random priming method of Feinberg and Vogelstein (1983) was employed to label double-stranded DNA molecules for subsequent use as a probe. The *AtSTP1* probe was generated following the PCR amplification of the template *STP1* cDNA. The probe did not hybridise to RNA from the *stp1* mutant. 50 ng of the *STP1* amplified DNA was heated to 100°C for 5 min to denature the DNA and subsequently chilled on ice. An appropriate volume of H_2O was added to the denatured DNA to achieve a final volume of 50 µl.

The following solutions were then added:

10µl	5 X oligonucleotide labelling buffer (OLB; recipe below)
2µl	BSA (10mg ml^{-1})
2µl	Klenow fragment DNA polymerase I
3µl	[α-^{32}P] dCTP (Amersham plc, Bucks, UK)

1 X OLB: 50 mM Tris-HCL pH 8.0, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.4 mM of each dATP, dGTP, dTTP, 250 mM HEPES, 1 mg ml^{-1} random priming hexanucleotides and pH adjusted to pH 6.6 with NaOH.

The labelling reaction was incubated at 37°C for 45 min. Unincorporated nucleotides were removed by filtration through a G50 Nick Column (Amersham plc, Bucks, UK).

2.2.6 Hybridisation of radio-labelled probes to membrane-bound RNA

RNA-bound membranes were incubated in 30 ml of pre-hybridisation buffer [4 X SSPE, 20 mM Tris-HCl pH 7.6, 1% (w/v) SDS, 0.2% (w/v) dextran sulphate and 2 X Denhardts solution (0.04% (w/v) Ficoll type 400 (Sigma-Aldrich, Gillingham, Dorset, UK), 0.04% (w/v) polyvinylpyrrolidone, 0.04% (v/v) BSA (fraction 5)] for at least 3 h at 65°C. The radio-labelled DNA probe was heated to 100°C for 5 min to ensure the DNA was denatured, prior to the addition of the probe to the pre-hybridisation buffer. The RNA-blots were incubated in the hybridisation buffer for approximately 16 h at 65°C.

To remove non-specifically bound radionucleotides the membranes were subsequently washed as follows:

4 X SSPE, 1% (w/v) SDS	65°C for 20 min X 2
2 X SSPE, 0.5% (w/v) SDS	65°C for 20 min X 2

After washing, the RNA-blots were wrapped in cling film.

2.2.7 Detection and quantitation of mRNA transcripts

The RNA-blots were exposed to Kodak Biomax X-ray film (Eastman Kodak company, Herts, UK) in autoradiography cassettes containing intensifying screens. The cassettes were stored at –70°C for several days. The X-ray films were subsequently developed with a Kodak X-Omat developer (Eastman Kodak company, Herts, UK). Alternatively, RNA-blots were exposed to a phosphor-imaging screen and the mRNA was subsequently quantified by relative intensity with a Storm 860 phosphor-imager (Molecular Dynamics, Sunnyvale, CA, USA).

2.3 DNA isolation and analysis

2.3.1 Small scale extraction of plasmid DNA

The plasmid DNA from 5 ml of *E. coli* culture was isolated using a DNA mini-prep kit (Qiagen Ltd, Crawley, West Sussex, UK). The kit allowed the simultaneous processing of a high number of samples. The protocol was followed according to the manufacturer's handbook.

2.3.2 Horizontal gel electrophoresis of DNA

In most cases, DNA samples were analysed by electrophoresis through 1% (w/v) agarose gels using 0.5 X TBE (2.25 mM Tris-Borate, 0.05 mM EDTA (pH 8.0)) as a buffer. For cloning purposes, gels were prepared from low-melting-point agarose type IV at 0.8% (w/v) with 1 X TAE buffer (4 mM Tris-HCl, 2 mM sodium acetate, 0.1 mM EDTA, adjusted to pH 8.2 with glacial acetic acid). The agarose gels also included ethidium bromide at a final concentration of 0.5 μg ml^{-1}.

DNA samples were mixed with 4 volumes of loading buffer (15% (w/v) ficoll 400, 250 mM EDTA, 0.04% (w/v) bromophenol blue and 0.04% (w/v) xylene cyanol FF) to

DNA, and subsequently loaded into the wells of the agarose gel. A 1 Kb ladder (Promega, Southampton, UK) was used as a size marker.

2.3.3 Analysis of DNA

The analysis of isolated DNA and plasmids by PCR and restriction enzyme digestions were conducted by standard protocols as stated in Sambrook *et al.*, (1989).

2.4 DNA cloning and generation of reporter constructs

2.4.1 Molecular biology techniques

The molecular biology techniques used in the construction and testing of the reporter construct were conducted as stated in Sambrook *et al.*, (1989). These include PCR, restriction enzyme digests, the extraction of DNA from agarose gels, dephosphorylation of DNA and DNA ligation.

2.4.2 Oligonucleotides used in the PCR amplification of *STP1* DNA

M13 Reverse: 5' AGC GGA TAA CAA TTT CAC ACA GG 3'

M13 Universal: 5' CCC AGT CAC GAC GTT GTA AAA CG 3'

STP1PROM: 5' GCA G**CC ATG G**CT TTT ATC CTA AAC A 3'

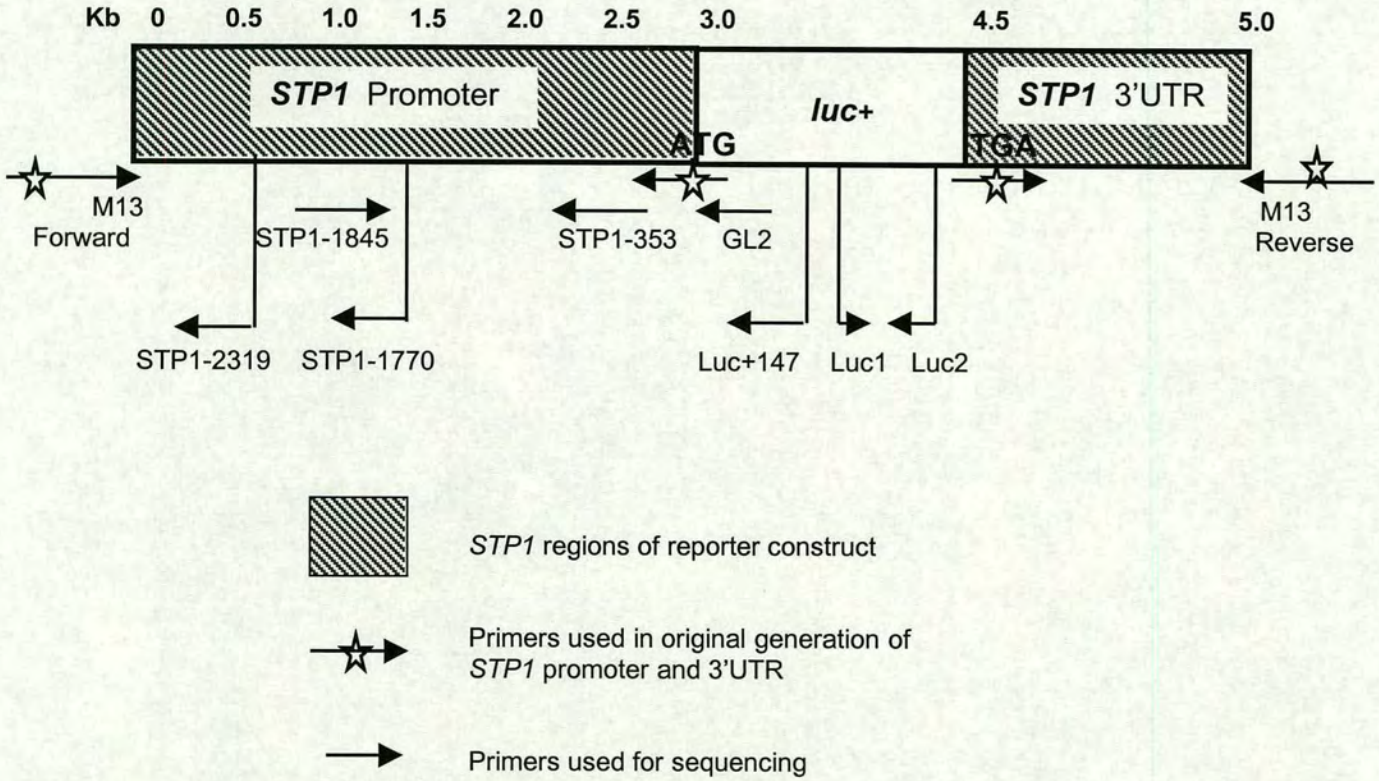
The *Nco* I site is highlighted

STP1END: 5' AAC **TCT AGA** TGT TTG ATT TAT CAT T .3'

The *Xba* I site is highlighted

A series of oligonucleotides priming in the *STP1* Promoter and the luciferase coding region (figure 2.1) was used in the sequencing of the reporter construct (designed by Dr S. Sherson, ICMB, University of Edinburgh, UK).

Figure 2.1 **Diagrammatic representation of the *STP1* Prom::*luc*$^{+}$::3'UTR construct and the priming sites of the oligonucleotides used to generate and sequence the construct.**



2.4.3 Strategy for the generation of *STP1* promoter::*Luc*$^+$::*STP1* 3'UTR

2.8 Kb upstream of the putative *STP1* ATG (start codon) representing the *STP1* promoter and 450 bp downstream of the putative *STP1* TGA (stop codon) representing the *STP1* 3'UTR were amplified by PCR. The template DNA was pBluescript containing cloned genomic *STP1* (N. Sauer, University of Erlangen, Germany). The *STP1* promoter was amplified using the primers STP1PROM & M13 forward and the *STP1* 3'UTR using STP1END & M13 reverse (section 2.4.2). The *STP1* primers were designed with specific homology to the *STP1* cDNA, with additional nucleotides representing the recognition sequence for particular restriction enzymes at the 5' end of the oligonucleotides. In the STP1PROM primer an *Nco* I restriction site was designed to allow the ligation of the putative *STP1* ATG start codon with that of the *luc*$^+$ reporter ATG. In the STP1END an *Xba* I restriction site was designed subsequent to the putative *STP1* TGA stop codon.

The *STP1* 3'UTR was sub-cloned into PUC18 using blunt-ended ligation at the de-phosphorylated *Sma* I site. The *STP1* Promoter was sub-cloned into pGem T-easy vector (Promega, Bucks, UK).

STP1 3'UTR was subsequently excised from PUC18 with *EcoR* I and *Xba* I restriction enzymes, and ligated into pSP-*luc*$^+$ at the 3' terminal of the luciferase coding region. *STP1* Promoter was excised from pGem T-easy with *Spe* I and *Nco* I, and ligated into pSP-*luc*$^+$*::3'UTR* resulting in a transcriptional fusion of the ATG sites from the *STP1* Promoter and the *luc*$^+$ coding region. The ligation of the *Spe* I restriction enzyme site (*STP1* promoter) and the *Avr* II restriction enzyme site (pSP-luc$^+$) resulted in the generation of a re-cleavable *Bfa* I site) (diagrammatic representation of the *STP1* promoter::luc$^+$::*STP1* 3'UTR reporter construct is included in section 3.6.2).

The complete reporter construct was finally cloned into the pGreen 0179 binary vector, which confers hygromycin resistance *in planta*.

2.4.4 DNA sequencing

DNA sequencing was performed with a D-rhodamine terminator cycle sequencing kit (Perkin Elmer, Beaconsfield, Bucks, UK) according to manufactures instruction. The template DNA was prepared using a Qiagen mini-prep Kit (section 2.3.1). The sequencing reactions were fractionated on a Perkin Elmer ABI Prism 377 DNA sequencer by the ICMB sequencing service (ICMB, University of Edinburgh, UK).

2.5 Transformations

2.5.1 *E. coli* electro-competent cell production

A single colony of *E. coli* TOP 10 (Invitrogen, Paisley, UK) was grown overnight at 37°C in 5 ml LB medium. 500 ml LB medium was inoculated with the 5 ml overnight culture, and grown at 37°C for several hours. The *E. coli* culture was grown until an optical density of A_{600} 0.6-0.7 was reached. The culture was subsequently cooled on ice for 30 min, and centrifuged at 4,000 g for 15 min. The pellet was resuspended in half the original volume in ice-cold sterile 10% (v/v) glycerol and centrifuged for a further 15 min. The pellet was again resuspended in half the volume of glycerol and centrifuged. The pellet was finally resuspended in 1-2 ml 10% glycerol and stored in 40 µl aliquots at -70°C, upon freezing the samples in liquid nitrogen.

2.5.2 Electroporation of *E. coli*

E. coli was transformed by electroporation using a Biorad Genepulser (Biorad Laboratories, Hemel Hemstead, Herts, UK). Five microlitres of plasmid DNA was added to an aliquot of competent cells. The mixture was transferred to an electroporation cuvette and left on ice for 15 min. Electroporation was conducted as instructed by the manufacturer's handbook at 25 μF, 200 Ω, 1.80 kV. Following the electroporation, 1 ml LB was added directly to the cuvette and mixed with the cell suspension. The mixture was then transferred to microcentrifuge tubes and incubated at 37°C for 1 h. 200 μl of the cell suspension was plated onto agar plates containing the appropriate antibiotic and subsequently incubated at 37°C overnight.

2.5.3 *Agrobacterium tumefaciens* competent cell production

5 ml LB medium containing 100 $\mu g ml^{-1}$ rifampicin (rif), 2.5 $\mu g ml^{-1}$ gentamycin (gent) and 2.5 $\mu g ml^{-1}$ tetracyline (tet) was inoculated with a singe colony of *A. tumefaciens* GV3101 and grown at 28°C for 48 h. One hundred millilitres LB containing rif, gent and tet (concentrations as above) was inoculated with 1 ml of the *A. tumefaciens* overnight culture and grown for a further 12 h. At an $A._{680}$ 0.5-1.0 the culture was incubated on ice for 10 min and centrifuged at 1500 rpm for 2 min. The remaining cell suspension was transferred to sterile falcon tubes and centrifuged for 6 min at 3,000 rpm at 4°C. The pellets were resuspended in ice-cold 20 mM $CaCl_2$ and stored in 200 μl aliquots at –70 °C.

2.5.4 Heat shock transformation into *Agrobacterium tumefaciens*

Five micrograms of plasmid DNA in 10 μl of water was added to 200 μl of competent *Agrobacterium tumefaciens*. The cell suspension was frozen in liquid nitrogen and subsequently thawed at 37°C for 5 min. The cell suspension was incubated at 30°C

of 2-4 h prior to plating on LB agar containing appropriate antibiotics. The transformants were grown at 28°C for several days.

2.5.5 *Arabidopsis thaliana* transformation

Agrobacterium tumefaciens-mediated *in planta* transformation of *Arabidopsis* was performed according to Bechtold *et al.,* 1993. The inflorescences of a flowering *Arabidopsis* plant were 'dipped' in an *A. tumefaciens* culture, previously transformed (section 2.5.4) with the pGreen 0179-reporter construct. The seeds from dipped plants were collected and screened for potential transformants on hygromycin-containing plant medium.

2.6 Expression analysis of transgenic plants

2.6.1 Luciferase imaging

Arabidopsis plantlets grown on vertical ½ MS plates were pre-treated with a water-based solution containing 5 mM beetle luciferin (Promega, Southampton, UK), 0.01% Triton X-100. The solution was brushed onto the plantlets 12 h prior to imaging and again 5 min before imaging. A charge-coupled device (CCD) imaging camera (Andor Technologies, Belfast, N. Ireland, UK) that detects low light was used, with Andor software to count the photon emissions from luciferase activity.

2.7 Sugar analysis

2.7.1 Uptake of radio-labelled 3-O-methyl glucose into *Arabidopsis*

Approximately 25-35 mg *of Arabidopsis* plantlets, previously grown on vertical ½ MS plates, were collected in microcentrifuge tubes. Plantlets were immersed in 200 μl of

Gamborg basic salt solution (full-strength Gamborg basic salts, 0.5% (w/v) MES and pH adjusted to pH 5.7 with NaOH). 0.2 μCi of α[^{14}C]3-O-methyl glucose, (specific activity 55.2 mCi/mmol) in 10 μl was added to the plantlet suspension and incubated for 30 min. After removal of the incubation medium plantlets were washed 5 times with 1 ml ice-cold Gamborg solution (same as above) containing 1 mM non-radioactive 3-O-methyl glucose. The [^{14}C]3-O-methyl glucose was extracted from either whole plantlets, or from the shoots and the roots following their separation. The washed tissue or plantlet samples were incubated in 1 ml 80% (v/v) ethanol at 4 °C for 1-12 h hours. Samples were subsequently heated at 56°C for 30 min, until the plantlets/tissue appeared colourless. The extract was then added to 10 ml Optiscint 'Hisafe' aqueous scintillant (Fischer Chemicals, Loughborough, Leic, UK) and counted twice for 4 min on a Beckman-Coulter LS 6500 scintillation counter (High Wycombe, Bucks, UK).

2.7.2 Autoradiography of *Arabidopsis* plantlets after uptake of [^{14}C]3-O-methyl glucose

Approximately 35-45 mg plantlets were collected from ½ MS plates and immersed in 1 ml of Gamborg basic salt solution (full-strength Gamborg basic salts, 0.5 % (w/v) MES and pH adjusted to pH 5.7 with NaOH). One microCurie of α[^{14}C]3-O-methyl glucose (specific activity 55.2 mCi/mmol) was added to the Gamborg solution. Plantlets were subsequently incubated in the radio-labelled sugar solution for 15 min. The plantlets were then removed from the incubation medium into 500 ml of ice-cold distilled water. Plantlets were washed a further 4 times in 500 ml ice-cold distilled water. The plantlets were then arranged in autoradiography cassettes. Excess water was removed using a tissue to gently blot the plantlets. Plantlets were then covered with cling-film or thin plastic and exposed to Kodak Biomax X-ray film (Eastman Kodak company, Herts, UK) at – 80°C for at least 4 weeks. The films were

subsequently developed with a Kodak X-Omat developer (Eastman Kodak company, Herts, UK).

CHAPTER THREE: Regulation of the *STP1* gene in plantlets grown and maintained in continuous light

3.1 Introduction

Generally, sugar depletion (famine response) enhances the expression of genes involved in photosynthesis, reserve mobilisation and carbohydrate-export processes, whereas abundant sugar resources (feast response) induce those genes involved in storage and utilisation (Koch, 1996). The main focus of this chapter is the effect of exogenous glucose upon the synthesis and activity of the STP1 monosaccharide transporter in plantlets maintained under different light regimes.

The analysis of several sugar-response mutants has identified cross talk between sugar signalling and that of ABA, ethylene and light. It appears that many sugar-responsive genes are dependent on ABA signalling pathways to regulate gene expression effectively. In this chapter the expression of the *STP1* gene is investigated in two such mutants; the *aba2* and *abi4* mutants that are defective in the synthesis and response of ABA respectively.

Light perception by photoreceptors can result in the modulation of genes involved in plant development and metabolism. Light regulation is important for optimising the photosynthetic production of sugars and storage of sugars as starch during the day, with the utilisation of starch reserves during the night. Moreover, genes that are induced by darkness and carbohydrate limitation (*din* genes) have been implicated in range of essential processes, such as the glyoxylate cycle (Graham *et al.,* 1994), nitrogen metabolism (Lam *et al.,* 1994), senescence-associated nutrient salvage (Fujiki *et al.,* 2001), and cell elongation (Braam *et al.,* 1997). The regulation of several *din* genes is thought to be dependent on phytochrome perception. In this chapter the dark-induction of the *STP1* gene is investigated. The role of light in modulating the synthesis and activity of the STP1 transporter is addressed. The requirement of phyA and phyB photoreceptors in transducing the light signal is also

considered, by monitoring the response of the *STP1* gene in *phyA*, *phyB* null mutants.

3.2 *STP1* gene expression under light and dark conditions

3.2.1 Aims

In previous studies the expression of the *STP1* gene is different under light and dark growth conditions (Hemmann, 2000). identified an accumulation of *STP1* transcripts under dark treatment relative to that in light-grown seedlings. Therefore the importance of light-dark transition in *STP1* gene regulation is investigated below. The expression of the *STP1* gene was monitored in Col0 plantlets that were grown in constant light (LL), transferred to constant dark (DD), and then returned to white light.

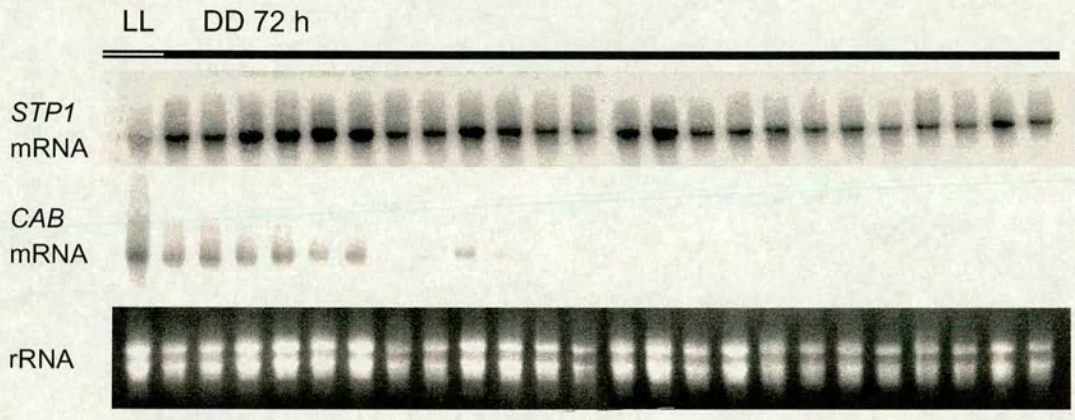
The expression of the *STP1* gene is also compared to that of the chlorophyll a/b binding protein (*CAB*) gene, which also responds to sugars (Sheen, 1990).

3.2.2 Light to Dark transition

Col0 plantlets were grown in continuous light for 10 days. Plantlets were then transferred to DD and shoot samples were harvested every 3 h for 72 h (0 h time point was harvested prior to the dark incubation). The *STP1* and *CAB* transcript levels are displayed as a RNA gel-blot (figure 3.2.1A) and upon subsequent quantification by phosphor-imaging (figure 3.2.1B).

The quantity of *STP1* transcripts is low in light-grown plantlets, relative to that of *CAB* transcripts (figure 3.2.1B; 0 h time-point). *STP1* transcripts accumulate within 3 h incubation in the dark, peaking after 15 h darkness. The absolute level of *STP1*

A: *STP1* and *CAB* transcript levels following RNA gel-blot analysis



B: The relative intensities of the *STP1* and *CAB* transcript levels following quantification by phosphor-imaging of the RNA gel-blot

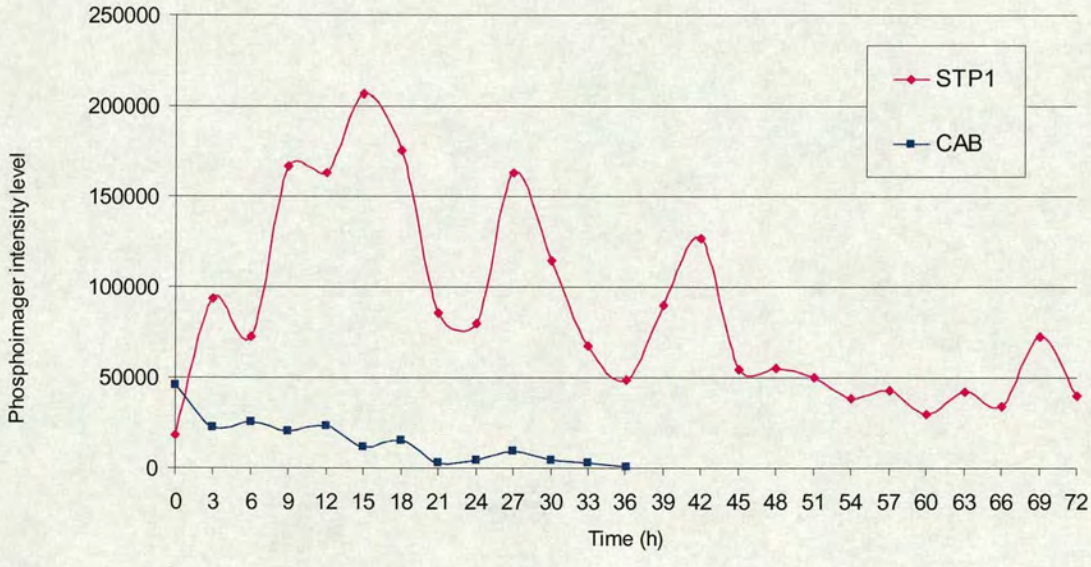


Figure 3.2.1 The *STP1* and *CAB* transcript levels in light-grown plantlets and following the subsequent incubation in darkness for 72 h

Plantlets grown for 10 days in continuous light were transferred to darkness for 72 h. The expression of the *STP1* (pink) and *CAB* (blue) genes was monitored in shoot tissue, harvested every 3 h.

transcripts falls during the remainder of the dark incubation. The *STP1* transcript level fluctuates greatly during the 72 h dark; the transcripts appear to cycle but the length of the cycles are not conserved to a particular time period.

The opposite expression profile is observed upon analysis of the same RNA gel-blot, following hybridisation with a *CAB* specific probe (figure 3.2.1). *CAB* transcripts accumulate in the light and diminish upon transfer of the plantlets to the dark. *CAB* transcripts are not detectable after 36 h in the dark.

The expression of the *STP1* gene was also monitored in light-grown plantlets following a 5 h dark-treatment and re-introduction to the light (figure 3.2.2). The quantity of *STP1* transcripts is low in light-grown plantlets (LL) and increases 10-fold after 5 h darkness. The subsequent exposure of dark-treated plantlets to white light results in a marked reduction in the level of *STP1* transcripts within 5 min and a return to basal levels (relative to LL; figure 3.2.2) within 3 h. Figure 3.2.2

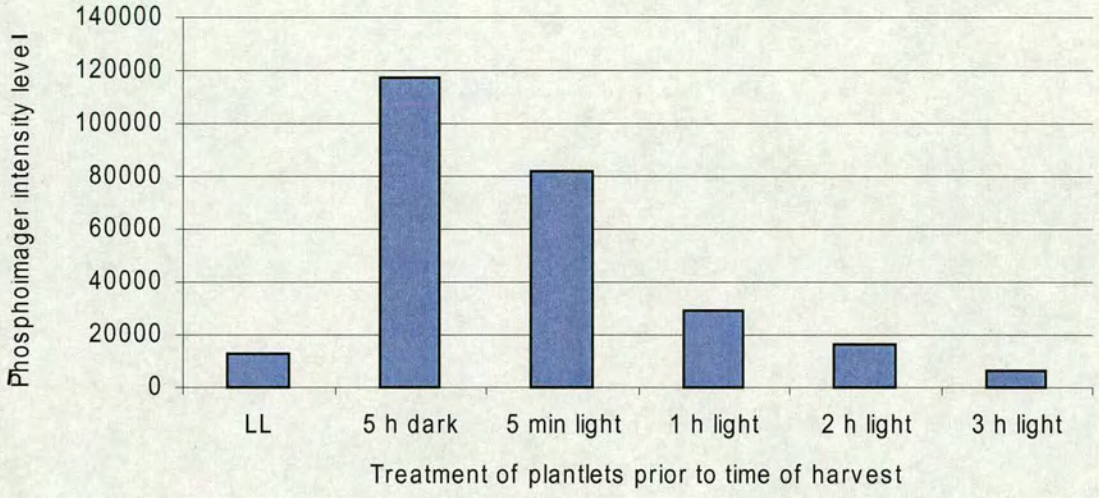


Figure 3.2.2 The effect of white light upon the *STP1* gene expression in dark-treated plantlets

Plantlets grown in continuous light for 10 days were transferred to the dark for 5 h. The expression of the *STP1* gene was monitored in shoot tissue upon subsequent treatment of the plantlets with white light for 3 h.

3.3 The response of the *STP1* gene to sugars

3.3.1 Aims

The expression of the *STP1* gene is repressed in seedlings treated with metabolisable sugars (Hemmann, 2000). To investigate sugar-response of the *STP1* gene further, light grown plantlets were treated with various concentrations of glucose under both light and dark conditions; the time and concentration dependence of *STP1* glucose response was also monitored.

Several sugar-sensing mutants are allelic to *aba2* and *abi4* loci. Therefore, it is thought that the ABA signalling pathway is linked with sugar signalling. The *aba2* and *abi4* mutants are defective in the synthesis and response of ABA respectively; these mutants are used in this study to address the regulation of *STP1* gene expression in the absence of ABA signalling.

Furthermore, while conducting such experiments, the actual process of incubating plantlets in sugar solution seemingly resulted in an alteration of *STP1* gene expression, independently of the sugar-response. The regulatory factor(s) responsible for initiating such a response are also addressed.

3.3.2 The glucose response of the *STP1* gene under different light and dark conditions

The response of the *STP1* gene to a range of glucose concentrations (0-30 mM) was investigated under three different light regime treatments (figure 3.3.1). Light-grown plantlets were incubated with exogenous glucose for 3 h in the light (lanes 2-4) and the dark (lanes 5-7); plantlets were also dark-treated for 12 h prior to the 3 h dark and glucose incubation (lanes 8-10). *STP1* gene expression is low in light-grown plantlets (lane 1). The expression level is further reduced by exogenous glucose. A 3 h light incubation with 10 mM (lane 3) and 30 mM (lane 4) glucose reduces *STP1* transcript level relative to that without glucose (lane 2), in a concentration dependent manner. In addition, the quantity of *STP1* transcripts in plantlets incubated with water in the light (0 mM lane 2) appears to be higher than that in untreated plantlets (lane 1). This raises the possibility that the expression of the *STP1* gene may be regulated by mechanical stimulation, flooding, stress or oxygen deficiency (see section 3.3.6 for further investigation).

The simultaneous incubation of light-grown plants (lane 1) with exogenous glucose and darkness (lanes 5-7) investigates the effect of glucose during the initial induction of the *STP1* transcript accumulation, which results upon transfer of plantlets in the dark; whereas in plantlets incubated in the dark for 12 h prior to the addition of exogenous glucose (lanes 8-10), the effect of glucose upon accumulated *STP1* transcripts is investigated. Exogenous glucose represses the *STP1* transcript level in plantlets irrespective of the dark treatment. However, the repression of *STP1* transcripts during the 3 h 30 mM glucose treatment in plantlets not previously exposed to the dark appear to be repressed to a lesser extent, compared to that in

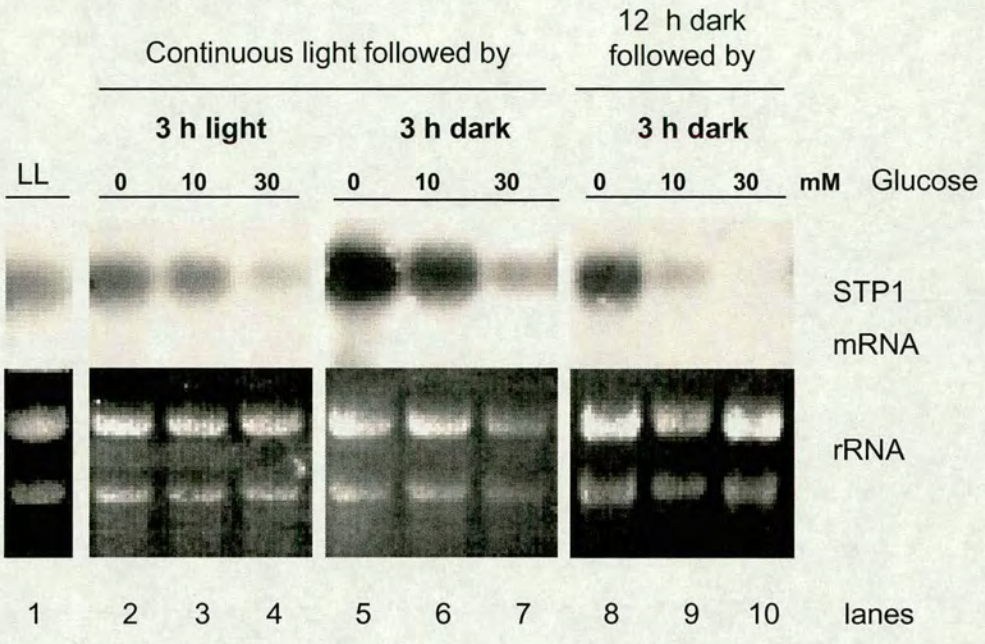


Figure 3.3.1 Glucose repression of *STP1* gene expression in *Arabidopsis* plantlets under different light and dark conditions

Plantlets were grown in continuous light for 10 days. One set of plantlets was then transferred to the dark for 12 h prior to sugar treatments. The *STP1* transcript level in light-grown and dark-incubated plantlets was monitored upon treatment with 0, 10 or 30 mM glucose in both light and dark conditions.

in plantlets previously dark-incubated (lanes 7 and 10 respectively). In this experiment the control samples were incubated with water, with the exception of light-grown plantlet tissue sample (lane 1). A shortcoming of this experiment is the absence of untreated plantlets as control samples during the experimental treatments.

The repression of *STP1* gene expression in dark-incubated plantlets is more apparent compared to that in light-incubated plantlets. This is likely to be because the *STP1* transcript level is appreciably higher in plantlets incubated in the dark relative to that in the light. Therefore, subsequent experiments include a dark incubation step, resulting in a raised expression level of the *STP1* gene, prior to glucose treatments.

3.3.3 The effect of various glucose concentrations upon *STP1* gene expression

The effect of a range of glucose concentrations (1-100 mM) upon *STP1* gene expression was investigated in dark-incubated Col0 plantlets (figure 3.3.2). Light-grown plantlets were incubated in the dark for 12 h, and subsequently treated for 1 h with 1, 3, 10, 30, 100 mM exogenous glucose, or were not treated (0 mM). The addition of 1, 3 and 10 mM glucose to plantlets maintained in the dark did not appreciably alter *STP1* gene expression (lanes 2, 3 and 4 respectively) relative to plantlets without glucose (lane 1). However, a reduction in the *STP1* transcript level was apparent upon treatment with 30 and 100 mM glucose (lane 5 and 6), the greatest repression was seen with 100 mM glucose (lane 6).

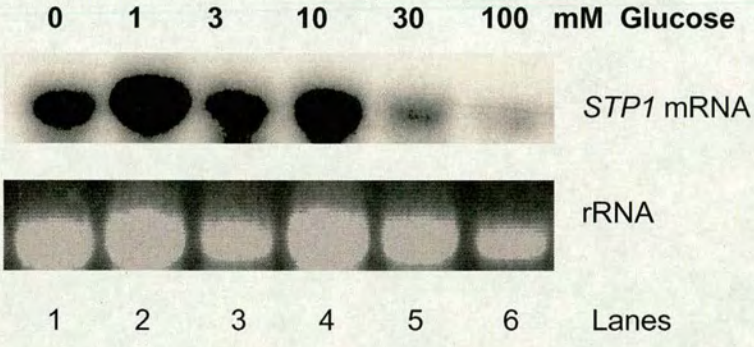


Figure 3.3.2 Repression of the *STP1* transcript level in plantlets treated with exogenous glucose, ranging in concentration from 0 to 100 mM glucose

Plantlets grown in continuous light for 2 weeks were transferred to the dark for 12 h. Dark-incubated plantlets were treated for 1 h with either 1, 3, 10, 30, 100 mM exogenous glucose, or not treated (0 mM). Whole seedlings were subsequently harvested, and the expression of the *STP1* gene following each treatment determined.

The shortcoming of this experiment is that the control for the exogenous glucose treatments (0 mM sample) was not incubated with water. However, this reveals an interesting facet of *STP1* gene expression as the amount of *STP1* transcripts in plantlets incubated with 1 mM glucose (lane 2) is higher than without glucose treatment (lane 1). It is possible that low concentrations of glucose increase *STP1* transcript levels. Alternatively, as the concentration of glucose is very low, it is possible that this increase in *STP1* transcripts is a result of an external factor regulating *STP1* gene expression; for example, mechano-stimulation, water induction, stress or lack of oxygen (refer to section 3.3.6 for more detail).

Furthermore, the *STP1* transcript level in plantlets incubated with 3 mM and 10 mM glucose (lane 3 and 4) is repressed compared to that observed with 1 mM glucose (lane 2), particularly when taking account of the under loading of RNA in lane 3. Thus demonstrating that low concentrations of D-glucose may repress the expression of the *STP1* gene.

3.3.4 A time-course of the glucose repression of *STP1* transcripts

Dark-incubated Col0 plantlets were treated with 3 mM exogenous glucose and samples were subsequently harvested every 30 min for 3 h (figure 3.3.3). A marked reduction in the dark-accumulated *STP1* transcript level (lane 1) results after 1 h (lane 3) and the reduction is greater after 3 h (lane 7) in plantlets incubated with 3 mM glucose. The incubation of plantlets with 3 mM glucose in this experiment is more effective at repressing the *STP1* transcript level compared to that seen in the previous experiment (figure 3.3.2). Slight variations in the glucose response of the *STP1* gene are evident throughout this report. This could be due to variations in the

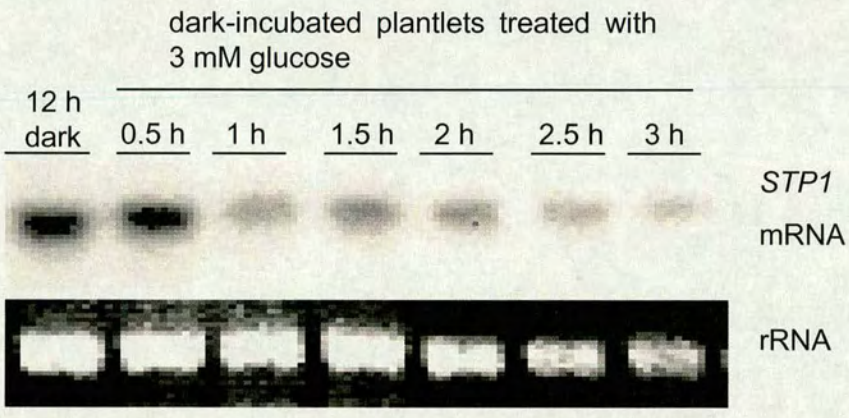


Figure 3.3.3 Repression of *STP1* gene expression by 3 mM glucose in Col0 plantlets

Plantlets were grown in continuous light for 1 week and transferred to the dark for 12 h. Dark-incubated plantlets were subsequently treated with 3 mM exogenous glucose and the *STP1* transcript level was monitored in shoot tissue every 30 min for 3 h.

manner in which plantlets were grown for each experiment; for example, plantlets may be exposed to varying degree of shading, distance from light source or mechanical stimulation.

3.3.5 The glucose response of the *STP1* gene in metabolic mutants

The glucose response of the *STP1* gene was investigated in 10-day-old Col0, *aba2* mutant and *abi4* mutant plantlets. Light-grown plantlets were incubated in the dark for 4 h and subsequently treated with 0, 3 and 30 mM glucose for 3 h. Shoot tissue was harvested at each stage and the *STP1* transcript level was visualised by RNA gel-blot analysis (figure 3.3.4). The dark accumulation of *STP1* transcripts and subsequent glucose repression is not apparently impaired in either of the mutants. This suggests that the glucose response of the *STP1* gene is independent of the ABA signalling pathway. However, further experimentation with exogenous ABA (Indole-3-Acetic Acid) is required to confirm this result.

3.3.6 Other regulatory factors that may modulate *STP1* gene expression

Previous experiments have shown that following the incubation of plantlets with water or low concentrations of exogenous glucose, an increase in the *STP1* mRNA level occurs (figures 3.3.1 and 3.3.2). The regulation of *STP1* gene expression described above is likely to be due to some external factor, such as mechano-stimulation, water induction, stress or lack of oxygen. To investigate the possibility of mechano-stimulation, light and dark incubated plantlets were deliberately stimulated mechanically (brushing and flicking of plantlets) and the *STP1* transcript level was monitored 4 h later (figure 3.3.5).

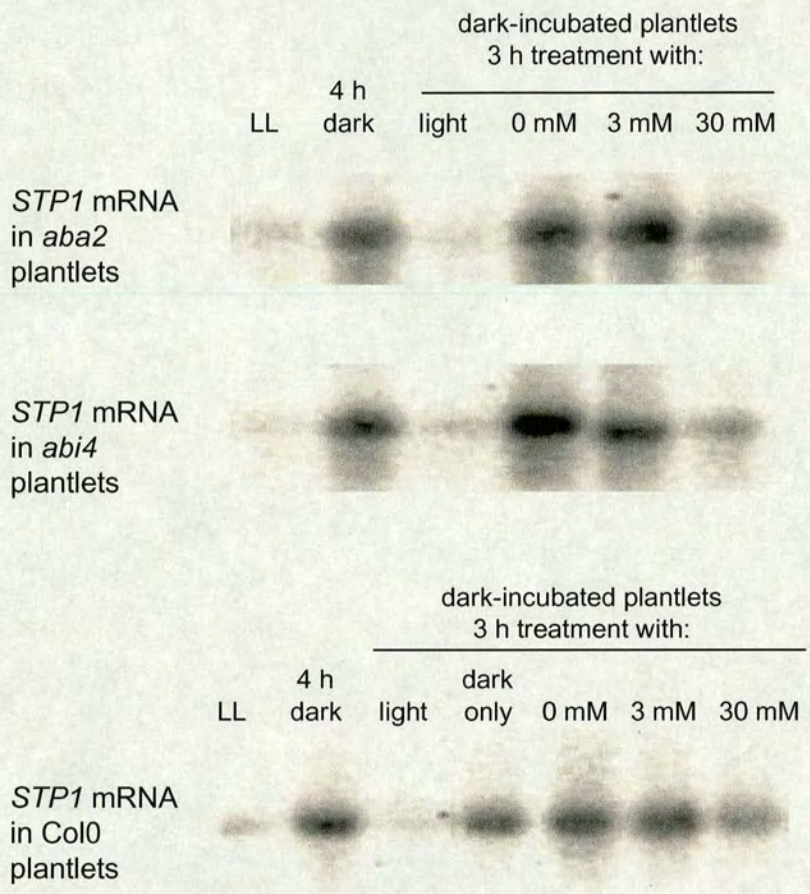


Figure 3.3.4 The response of *STP1* gene expression to sugar and light in wild-type and ABA synthesis and response mutant plantlets

Col0, *aba2* and *abi4* plantlets were grown in continuous light for 10 days and subsequently transferred to the dark for 4 h. Dark-incubated plantlets were then treated with light or 0, 3 and 30 mM glucose for 3 h. Upon the harvest of shoot tissue following the respective treatments of plantlets the *STP1* transcript level was monitored by RNA gel-blot analysis.

An extra sample was included due to a surplus of Col0 plant material, and the recent discovery of mechano-stimulation (see section 3.3.6); Col0 dark-incubated plantlets were maintained in the dark for a further 3 h without treatment.

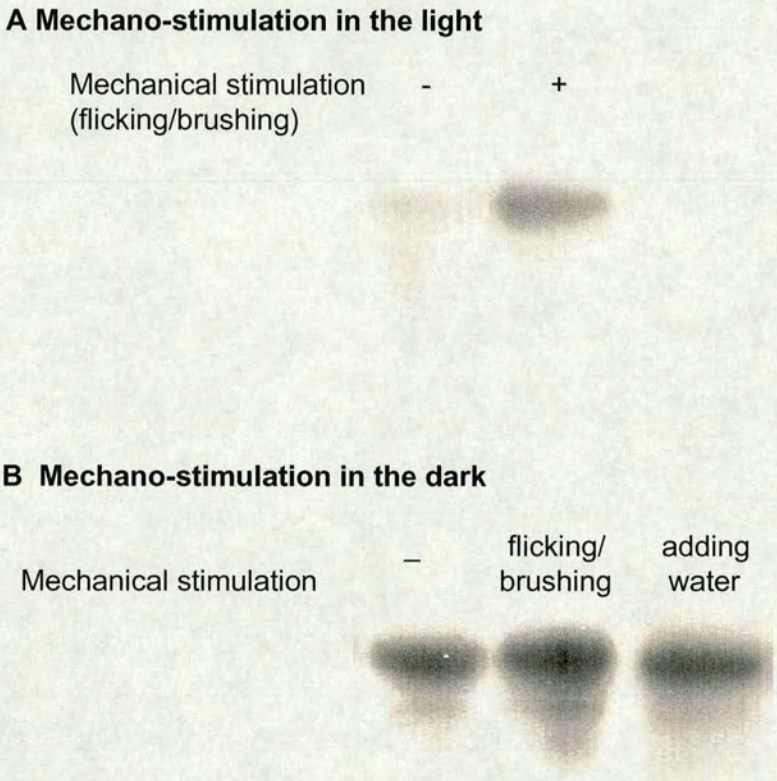


Figure 3.3.5 The effect of mechano-stimulation upon STP1 gene expression in plantlets under light and dark conditions

Plantlets were grown in continuous light for 2 weeks. The effect of mechanical stimulation upon the *STP1* transcript level in light-grown plantlets (A) and dark-incubated plantlets (B) is displayed. The *STP1* transcript level was monitored in the shoot tissue of treated and untreated plantlets.

The expression of the *STP1* gene in light-grown plantlets subjected to mechanical stimulation was monitored (figure 3.3.5A). Plantlets were deliberately stimulated mechanically by brushing and flicking the plantlets; shoot samples were harvested 3 h later. *STP1* transcripts accumulate to a greater extent in the shoots of plantlets that were subjected to mechanical stimulation (lane 2) compared to that observed in untreated plantlets (lane 1).

In addition, the shoot-specific *STP1* gene expression was monitored in dark-treated plantlets after further treatment with either brushing/flicking, water incubation or no treatment (figure 3.3.5B). There is a higher level of *STP1* transcripts when plantlets were deliberately stimulated mechanically (lane 2) compared to that seen with other treatments (lane 1 and 3). This suggests that *STP1* gene expression may be regulated by mechano-stimulation. The *STP1* transcript level in the water-treated sample (lane 3), appears to be slightly higher than that seen in the control sample (lane 1), but lower than that in the mechanically stimulated sample (lane 2). This may possibly be due to the agitation of plantlets that results upon the addition of water. Therefore, flooding of plantlets may inadvertently cause the mechanical stimulation of the plantlets, resulting in the induction of *STP1* gene expression, which is less effective than flicking and brushing the plantlets.

STP1 gene expression appears to be more responsive to mechano-stimulation in the shoots of plantlets that are incubated in the light. This is possibly due to the fact that the level of *STP1* transcripts in dark-incubated plantlets is higher and therefore further induction of the *STP1* gene does not result in a marked change in the transcript level. Whereas, *STP1* gene expression in the light is low, so the induction of *STP1* transcripts has the potential to be much greater. Alternatively, it may be the case that as *STP1* gene expression is lower in the light an increase in *STP1* transcripts is more visually apparent.

Moreover, data from the Stanford microarray database pertaining to *STP1* gene (clone ID 171P10T7) regulation is consistent with the accumulation of *STP1* transcripts as a result of mechanical stimulation. A 3-fold increase in *STP1* transcripts is reported upon gentle mechanical stimulation (http://genome-www4.stanford.edu/cgi-bin/SMD/spothistory.pl).

3.4 Light response of *STP1* gene expression

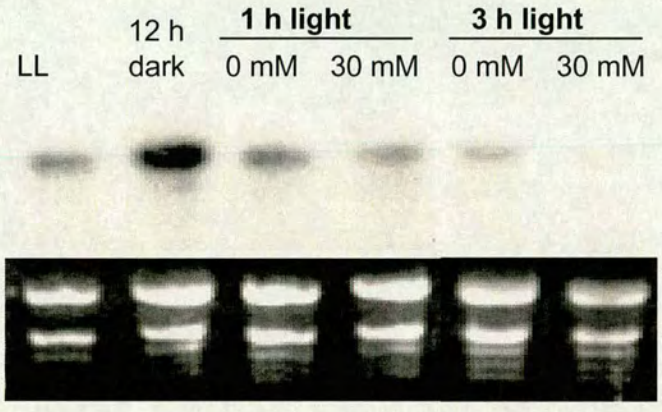
3.4.1 Aims

The expression of the *STP1* gene is induced in the dark (section 3.2). The effect of light upon the *STP1* transcript level is investigated further in this section. Experiments address whether the repression of the *STP1* gene by light acts in a similar manner to glucose regulation (section 3.3), and whether light and glucose act through the same signalling pathway. Furthermore, the requirement for the principle phytochromes (phyA and phyB) in modulating the expression of the *STP1* gene in response to light and/or sugar is also addressed.

3.4.2 The additive effect of light and sugar upon *STP1* gene expression

The expression of the *STP1* gene is repressed by exogenous glucose in the light (section 3.3.1). To investigate whether the effect of glucose and light is additive, plantlets were dark-incubated for 12 h, prior to the treatment of plantlets with or without 30 mM glucose under light (figure 3.4.1A) and dark conditions (figure 3.4.1B). The *STP1* transcript level accumulates in light grown plantlets incubated in the dark for 12 h (figure 3.4.1A, lane 2). Upon transfer of dark-incubated plantlets to the light, with and without glucose, the *STP1* transcript level falls (lanes 3-6). The

A Light and Glucose



B Dark and Glucose

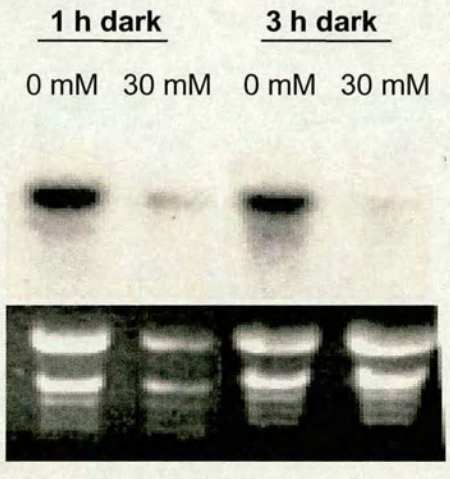


Figure 3.4.1 The effect of light and glucose upon *STP1* gene expression in dark-incubated plantlets.

Plantlets were grown in continuous light for 1 week and dark-incubated for 12 h. Plantlets were subsequently treated with or without 30 mM glucose in the light (A) or the dark (B). The *STP1* transcript level was monitored in whole plantlets 1 and 3 h after treatment.

greatest repression is seen in plantlets treated with both light and 30 mM glucose for 3 h (lane 6).

The *STP1* transcript level remains high in dark-incubated plantlets that are incubated in the dark for a further 1 h and 3 h (figure 3.3.1B, lanes 1 and 3 respectively). A reduction in the *STP1* transcript levels is observed in the plantlets that are dark-incubated with 30 mM glucose for a further 1 h and 3 h (lanes 2 and 4 respectively). This is consistent with previous glucose repression experiments (section 3.3).

3.4.3 The response of the *STP1* gene to light independently of sugar

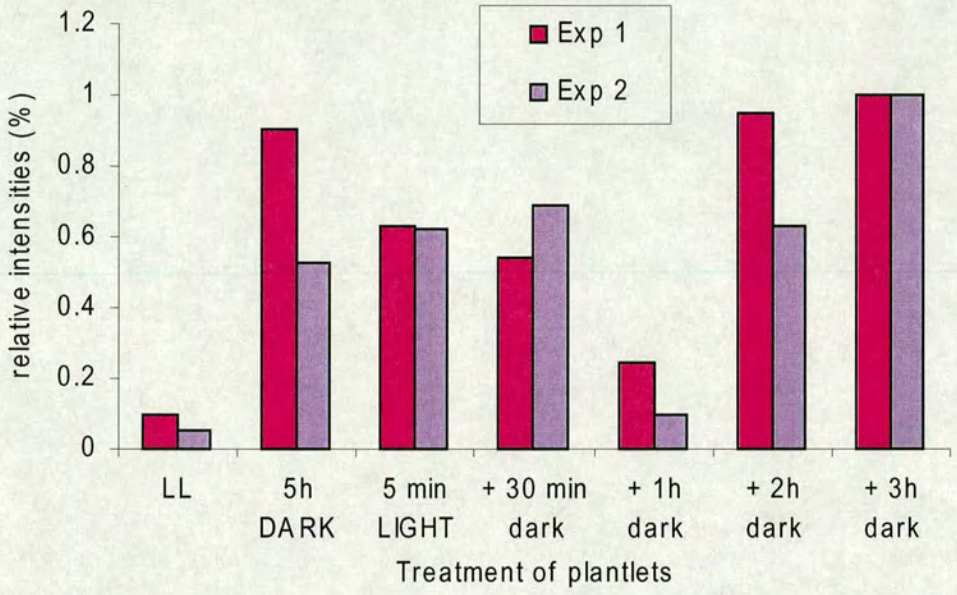
The repression of the *STP1* transcript level is greater in plantlets treated with both glucose and light relative to either treatment alone (figure 3.4.1). The experiment described below was designed to investigate whether the light response of the *STP1* gene is independent of the sugar response. Light-grown plantlets were dark-incubated for 5 h, exposed to white light (~200 μmol m^{-2} s^{-1}) for 5 min and subsequently re-introduced to the dark for a further 3 h. Shoot tissue was harvested every 30 min during the 3 h dark incubation (figure 3.4.2A). Control plantlets were either exposed to 5 min white light and maintained in the light for 3 h (figure 3.4.2B) or plantlets were dark-incubated for 5 h and maintained in the dark for a further 3 h without any light exposure (figure 3.4.2C).

STP1 transcripts accumulate in the shoots of dark-incubated plantlets and the subsequent interruption by 5 min white light results in a marked repression of *STP1* gene expression after 1 h in the dark (figure 3.4.2A). *STP1* transcripts in the shoots of plantlets that continue in the light following the 5 min light pulse are also

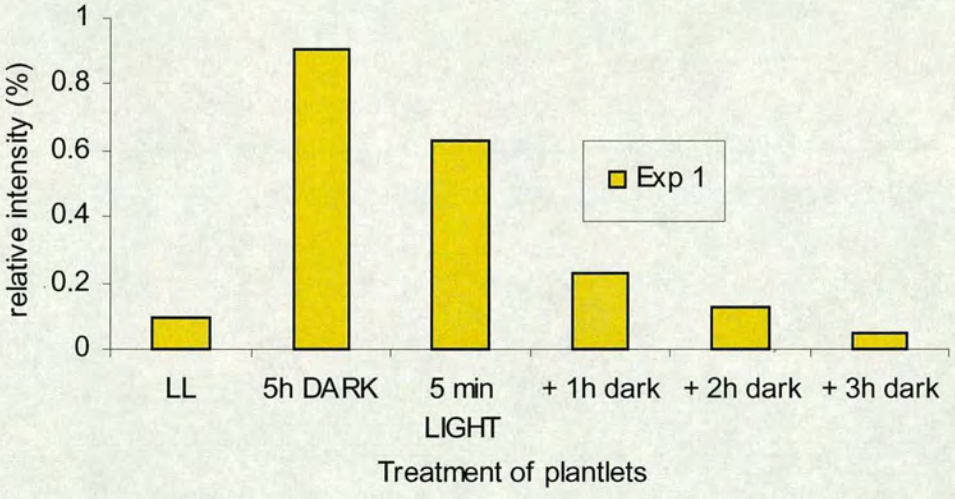
Figure 3.4.2 *STP1* gene expression in dark-incubated plantlets treated with 5 min white light. (next page)

Plantlets grown in continuous light for 10 days and dark-incubated for 5 h. One set of plantlets was maintained in the dark for 3 h (C). The remainder of dark-incubated plantlets were exposed to 5 min white light and either transferred to the dark (A) or maintained in the light (B) for a further 3 h. Shoot tissue was harvested and the *STP1* transcript levels were quantified by phosphor-imaging of the RNA gel-blot. The figure represents the data from 2 independent experiments; therefore the intensities of the *STP1* transcript level are displayed relative (%) to the lowest transcript level quantified in each experiment.

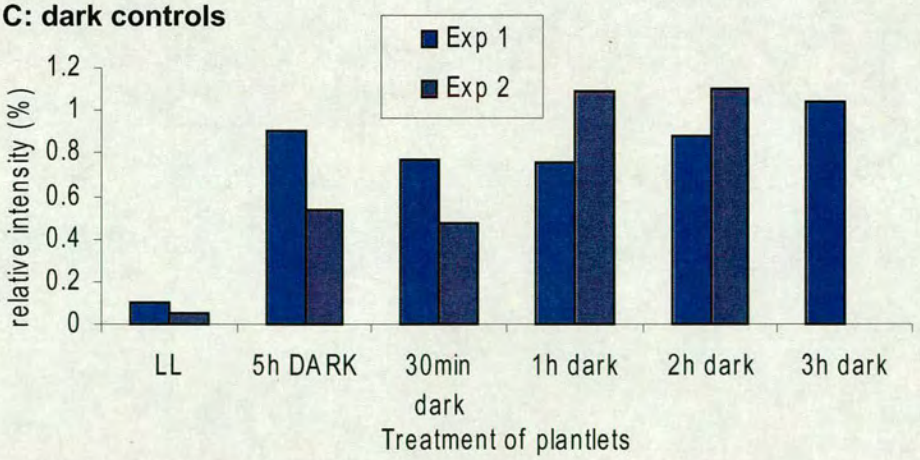
A: 5 min white light interruption of dark



B: light controls



C: dark controls



repressed after 1 h but unlike the *STP1* transcript level in A, the level continues to fall (figure 3.4.2B). The *STP1* transcript level in the shoots of the dark control plantlets (not exposed to light) remains high throughout the experiment (figure 3.4.2C). The repression of the *STP1* transcript level by 5 min light exposure is thought to be independent of sugar, because it is unlikely that the photosynthetic production of sugars would be sufficient in 5 min to cause such a response.

The reciprocal experiment shows the effect of a 5 min dark treatment upon the expression of the *STP1* gene in the shoots of light-incubated plantlets (figure 3.4.3). In plantlets either maintained solely in the light, or exposed to 5 min dark and returned to the light, the quantity of shoot-specific *STP1* transcripts remains low throughout. However, in plantlets that continued in the dark following the 5 min dark treatment *STP1* transcripts accumulate in shoot tissue after a 30 min dark. This suggests that 5 min dark is not sufficient to induce *STP1* gene expression.

3.4.4 The expression of the *STP1* gene in mutants defective in light signalling

In *Arabidopsis,* a number of light responsive genes require functional phyA and/or phyB for effective light signalling to occur (see introduction 1.8 for more detail). Therefore, to investigate the possible involvement of these photoreceptors in mediating the *STP1* light response, the expression of the *STP1* gene was monitored in the *phyA* and *phyB* monogenic null mutant plantlets, and in the *phyA phyB* digenic null mutant.

The glucose and light response of shoot-specific *STP1* gene expression in *phyA*, *phyB*, *phyA/B* mutant plantlets and in Col0 wild-type plantlets was monitored (figure 3.4.4). The expression of *STP1* gene in the mutants is similar to that seen in wild-

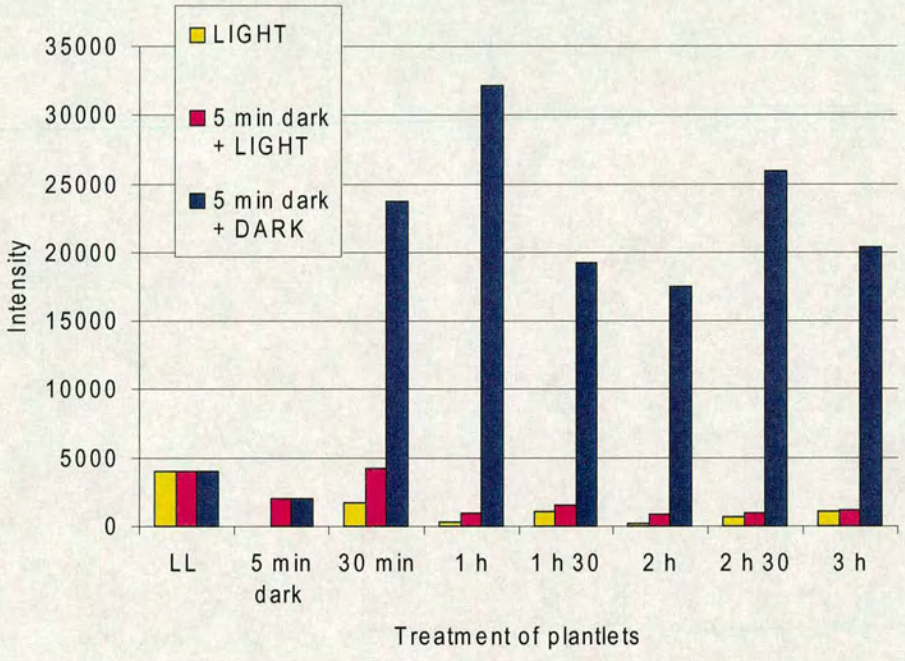


Figure 3.4.3 The effect of a a 5 min dark treatment upon the *STP1* gene expression in light-grown plantlets

Plantlets were grown in the light for 10 days. Plantlets were then either maintained in continuous light (yellow), or dark-treated for 5 min and subsequently incubated in the light (pink) or the dark (blue). The *STP1* transcript level was monitored over in all 3 light conditions, in shoot tissue harvested every 30 min for 3 h.

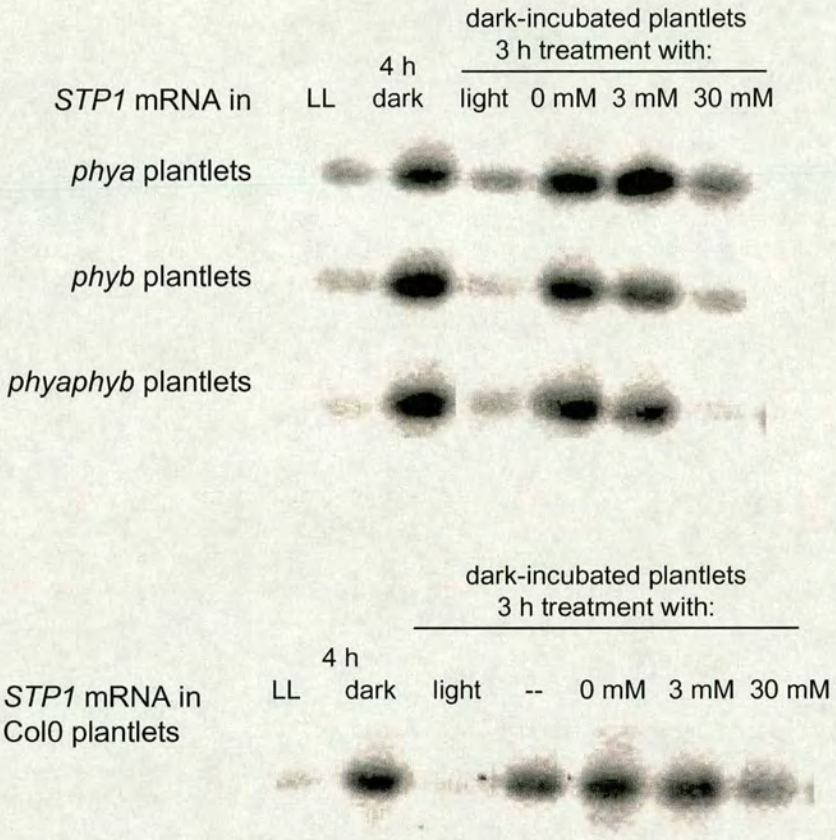


Figure 3.4.4 The response of *STP1* gene expression to sugar and light in wild-type and photoreceptor mutant plantlets

Col0, *phyA, phyB* and *phyA phyB* plantlets were grown in continuous light for 10 days and subsequently transferred to the dark for 4 h. Dark-incubated plantlets were then treated with light or 0, 3 and 30 mM glucose for 3 h. Col0 dark-incubated plantlets were also incubated in the dark for a further 3 h untreated (--). Upon the harvest of shoot tissue following the respective treatments of plantlets the *STP1* transcript level was monitored by RNA gel-blot analysis.

type plantlets. The quantity of *STP1* transcripts is low in the shoots of light-grown plantlets (lane 1). An accumulation of *STP1* transcripts is seen in the shoots of plantlets dark-incubated for 5 h (lane 2). Subsequent treatment of dark-incubated plantlets with light (lane 3) or 30 mM glucose (lane 6) results in a reduction of the *STP1* transcript level. The repression is greatest in the shoots of light-treated plantlets relative to that in the shoots of glucose-treated plantlets. The treatment of plantlets with 3 mM glucose (lane 5) has a negligible effect upon the *STP1* transcript level compared to that in water-incubated plantlets (lane 4). In *phyA* mutant plantlets, the amount *of STP1* transcripts in the shoot tissue of 3 mM glucose-treated plantlets is higher than that observed in water-treated plantlets (lane 5 and lane.4 respectively). The strongest repression of the *STP1* transcript level is seen in the shoots of *phyA/B* digenic mutants following 30 mM glucose treatment for 3 h. This experiment needs to be repeated before any significance can be attributed to such differences.

This experiment suggests that functioning phyA and phyB photoreceptors are not required for the repression of dark-accumulated *STP1* transcripts by glucose and light. In fact, a stronger repression of *STP1* gene expression by glucose may be evident in the absence of phyA and phyB.

More work is required to identify the photoreceptor(s) required to transduce the *STP1*-light response. However, these experiments do not completely eliminate the requirement for phyA and phyB, as the repression of *STP1* transcripts was only monitored in plantlets under white light. The effect of R and FR light, the active wavelengths for phyA and phyB respectively, in transducing the *STP1* light response in the null mutants may be more revealing.

3.5 The uptake of [^{14}C] 3,O-methyl glucose in light-grown plantlets

3.5.1 Aims

The uptake of [^{14}C] 3-O-methyl glucose (3-OMG) by *Arabidopsis* wild-type and *stp1* mutant seedlings indicate that STP1 is the major monosaccharides transporter in light-grown seedlings (Sherson *et al.,* 2000). In this section, the effect of light and glucose upon the transport activity of [^{14}C] 3-OMG in Col0 and s*tp1* mutant plantlets is investigated. The methodology of 3-OMG uptake assays is described in 2.7.

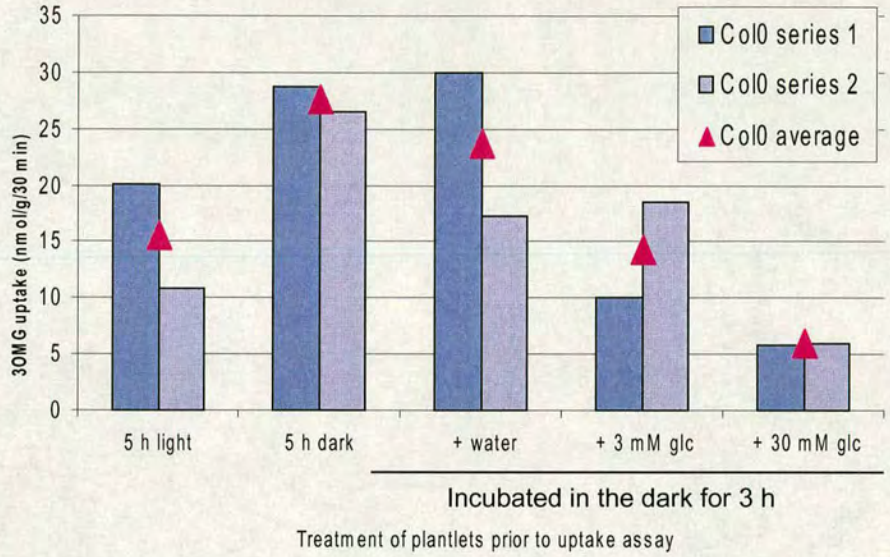
In the uptake experiments described below, only Col0 wild-type plantlets are used, which is the *Arabidopsis* ecotype used in all the experiments in this chapter. However, the *stp1* mutant is Ws ecotype; the Ws wild-type has not been used in the uptake studies described below, due to time constraints. However, more detailed experimentation with Col0, Ws and *stp1* mutant plantlets, grown in a diurnal growth regime, is reported in chapter 4. The results suggest that the gene expression and transport activity of STP1 is similarly regulated in both wild-type ecotypes (section 4.3).

3.5.2 The effect of glucose and light on 3-OMG transport activity

Light-grown plantlets were dark-incubated for 5 h and subsequently treated with water, glucose or light for a further 3 h; at each stage of the experiment the rate of [^{14}C] 3-OMG uptake was determined by both Col0 (figure 3.5.1A) and *stp1* mutant plantlets (figure 3.5.1B).

The average 3-OMG uptake by dark-incubated Col0 plantlets is almost double that seen in light-grown plantlets (figure 3.5.1A). Incubation of plantlets in the dark with 3 mM or 30 mM exogenous glucose for a further 3 h resulted in a reduction in 3-OMG uptake by 40 % and 60 % respectively, relative to that seen in water-treated

A: Rate of 3OMG uptake by Col0 plantlets



B: Rate of 3OMG uptake by *stp1* mutant plantlets

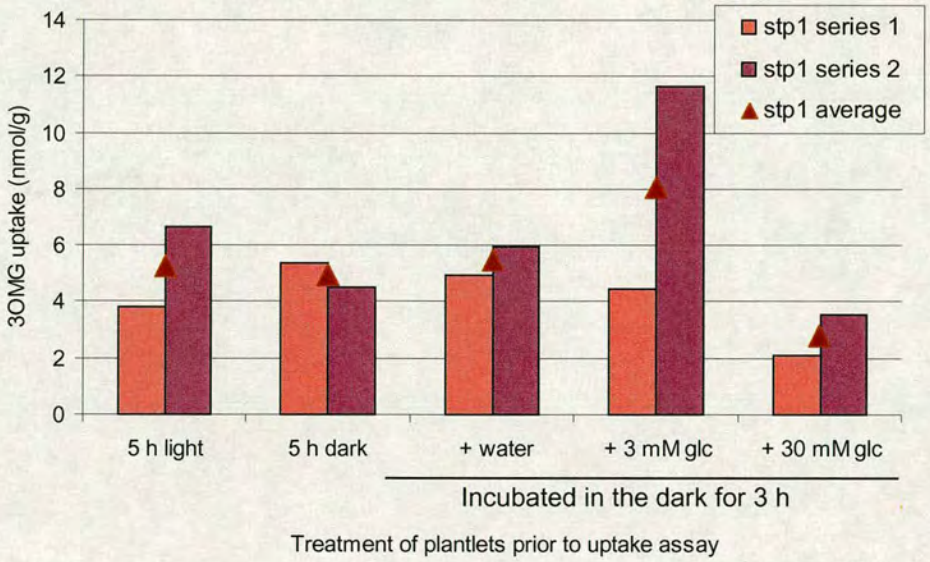


Figure 3.5.1 The effect of glucose upon the rate of [^{14}C]3-O-methyl glucose in wild-type and *stp1* mutant plantlets

Col0 and *stp1* mutant plantlets were grown in continuous light for 2 weeks. Plantlets were incubated in the light or the dark for 5 h, at which point plantlets were collected and the rate of 3OMG uptake was measured. The remainder of dark-incubated plantlets were treated with 0, 3, and 30 mM glucose for a further 3 h; plantlets were subsequently collected for 3OMG uptake assay.

plantlets. Therefore, the addition of exogenous glucose to wild-type plantlets reduces the STP transport activity of 3-OMG, in a manner consistent with the glucose response of the *STP1* transcript level (section 3.3).

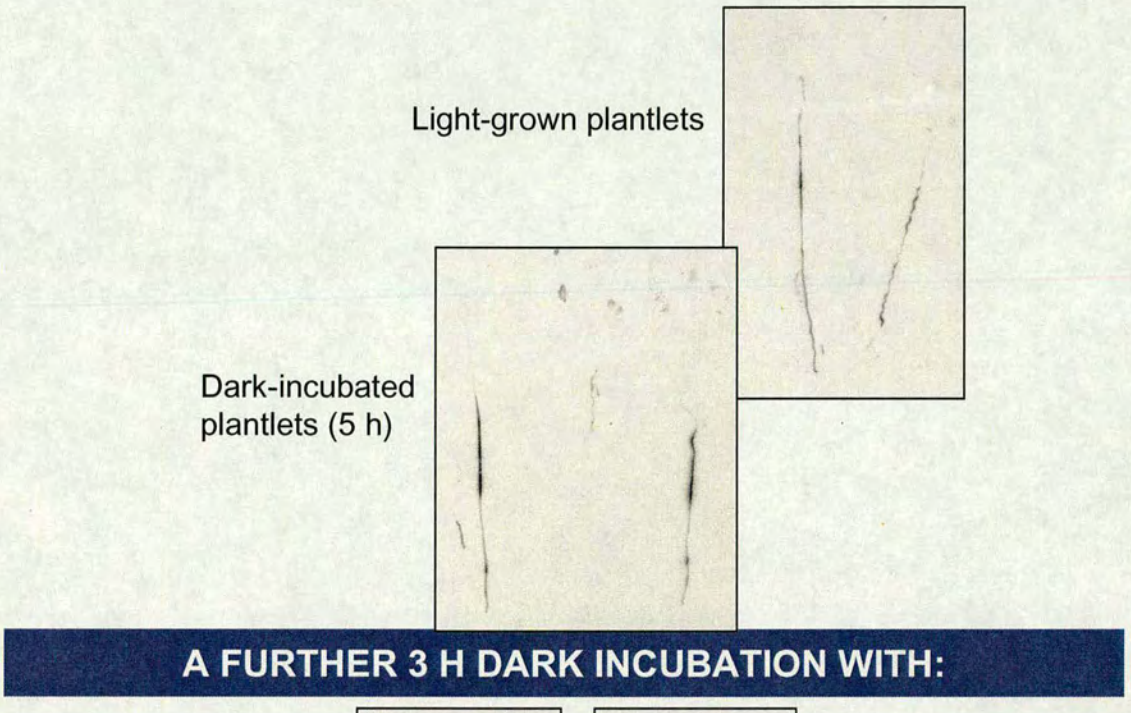
The average rate of 3-OMG uptake by the *stp1* mutant plantlets is similar, irrespective of the treatment administered (figure 3.5.1B). Although, in plantlets incubated with 30 mM exogenous glucose for 3 h the rate of 3-OMG uptake was reduced by approximately 50 % relative to that seen in water-treated *stp1* plantlets. It is possible that the synthesis and/or activity of one or more of the remaining STPs are also regulated by glucose.

The uptake of 3-OMG by the *stp1* mutant is reduced by ~70 % in the light and ~80 % in the dark, compared to that by wild-type plantlets in the same conditions. This data is consistent with STP1 transport activity accounting for the majority of the 3-OMG uptake in Col0 plantlets.

Autoradiography of [^{14}C] 3-OMG uptake by Col0 plantlets displays the spatial distribution of STP transport activity in the shoot and root tissue (figure 3.5.2). Light-grown Col0 plantlets were dark-incubated for 5 h and subsequently treated with water, glucose or light for a further 3 h. The [^{14}C] 3-OMG uptake assay was conducted, and plantlets were subsequently exposed to X-ray film. The rate of 3-OMG uptake by light-grown plantlets is predominantly localised to the root tissue (figure 3.5.2A). The 3-OMG uptake by the main root increases following the 5 h dark-incubation of plantlets (figure 3.5.2B). Treatment of dark-incubated plantlets with 30 mM glucose (E) and light (F) results in a decrease in the level of 3-OMG uptake, relative to that of water-treated plantlets (figure 3.5.2D). Also, the 3-OMG uptake by water-treated plantlets (D) is higher than that by untreated plantlets (C), particularly by the root tips. This suggests that mechano-stimulation may play a role

Figure 3.5.2 Autoradiography of 3-OMG uptake in dark-incubated plantlets treated with glucose and light displays the qualitative localisation of 3-OMG STP transport activity. (next page)

Col0 plantlets were grown in continuous light for 2 weeks and subsequently transferred to the dark for 5 h. Plantlets were then treated with water, 30 mM glucose or light for a further 3 h. The rate of 3-OMG in plantlets was determined qualitatively, following the exposure of assayed plantlets to x-ray film for 1 month.


Light-grown plantlets
Dark-incubated plantlets (5 h)
A FURTHER 3 H DARK INCUBATION WITH:
untreated
water
light
30 mM glucose

in the modulation of 3-OMG transport activity in wild-type plantlets. As this experiment only monitors 3-OMG transport activity in Col0, the results describe the total STP activity, rather than just STP1.

The quantitative uptake of 3-OMG by the shoots and roots, following the treatment of plantlets with dark and exogenous glucose has also been determined (figure 3.5.3). The 3-OMG uptake assay was performed with whole plantlets and the shoot and root tissue was separated prior to the extraction of radiolabelled sugars. The 3-OMG transport activity was investigated in light-grown plantlets, following a 24 h dark incubation, and subsequent transfer to the light for a further 3 h (figure 3.5.3).The 3-OMG transport activity in the shoots is similar to that seen in whole seedlings (figure 3.5.1), in that an increase in the rate of 3-OMG uptake occurs after plantlets were incubated in the dark. The opposite is observed in the roots, as a higher rate of 3-OMG uptake occurs upon the transfer of plantlets to the light compared to that seen by dark-treated plantlets. However, the rate of 3-OMG uptake by both shoot and root tissue decreases upon treatment with 30 mM glucose for 3 h.

There are a couple of differences between the qualitative (figure 3.5.2) and quantitative (figure 3.5.3) measurements of 3-OMG glucose by the shoots and the roots of Col0 plantlets. These may possibly be a result of variations in the external factors plantlets were exposed to during their growth, such as, differences in the shading, distance from the light or due to differences in the experimental procedures used (see section 2.7 for details), such as, the uptake assay was conducted for 15 min and 30 min for the measurement of qualitative and quantitative 3-OMG uptake respectively. Alternatively, plantlets may have been affected by mechanical stimulation to different extents, for example, the plantlets used for the autoradiography of 3-OMG uptake were vigorously agitated by washing with 500 ml

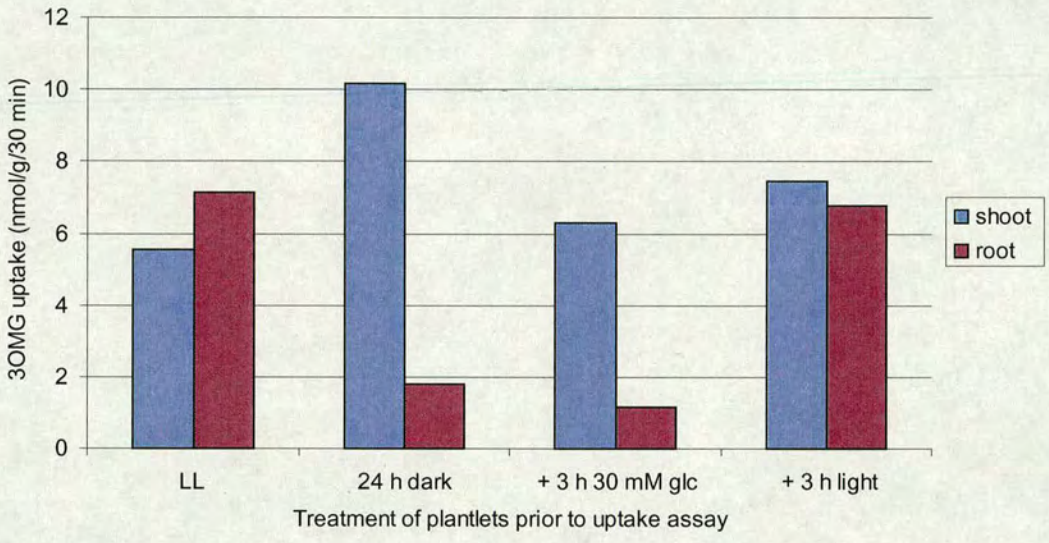


Figure 3.5.3 The effect of glucose and light upon the rate of [^{14}C]3-O-methyl glucose in both the shoots and the roots of wild-type plantlets

Col0 plantlets were grown in continuous light for 2 weeks and subsequently transferred to the dark for 24 h. Plantlets were then treated with 30 mM glucose or light for a further 3 h. The rate of 3OMG uptake was measured upon separation of the shoot and root tissue of assayed plantlets.

of water and the plantlets were then moved, and arranged in autoradiography cassettes.

3.6 The *STP1*-driven luciferase expression in light-grown plantlets

3.6.1 Aims

The fire-fly luciferase gene has been used in many studies to report the pattern of plant gene expression, such as, *CAB* (Miller and Kay, 1996) and *PC* (plastocyanin) (Dijkwel et al, 1996). It is the favoured reporter gene for identifying the rapid modulation of gene expression, as the encoded luciferase protein is relatively unstable. In the presence of exogenous luciferin, the encoded luciferase protein can catalyse a chemical reaction that results in the emission of photons, which in turn can be counted and imaged (Leeuwen *et al.,* 2000).

The *STP1* promoter::*luc*+::*STP1* 3'UTR reporter construct was generated primarily for use in the isolation of sugar-response mutants, which is presently in progress in the laboratory. However, this report displays preliminary data from the *Arabidopsis* transgenic lines containing the *STP1*-luciferase construct, following incubation in the light and the dark, and upon glucose treatment.

3.6.2 Strategy for the generation of *STP1 promoter::Luc$^+$::STP1 3'UTR*

The *STP1* Promoter and 3'untranslated region (3'UTR) were amplified from the full-length STP1 cDNA (Sauer *et al.,* 1990) by PCR. The 450 bp region downstream of the STP1 coding region (putative 3' untranslated region) was cloned into the pSP-luc+ vector (Promega). The *STP1* 3'UTR was ligated 10 bp downstream of the luciferase stop codon (TGA). Subsequently, the 2.8 Kb region of DNA upstream of the *STP1* coding region and including the putative ATG start codon was cloned into

the pSP-*luc*+::3'UTR vector. The *STP1* and *LUC*+ ATG start codons were fused together via the *Nco* I restriction site (figure 3.6.1). The *STP1* promoter::*luc*+::3'UTR reporter construct was cloned into the pGreen binary vector 0179 (Hellens *et al.,* 2000), which confers hygromycin resistance in plants (figure 3.6.2). The primers used for DNA amplification and the restriction sites used in the ligation steps are described in materials and methods (section 2.6)

3.6.3 The glucose response of *STP1*-driven luciferase expression

Analysis of *Arabidopsis* plantlets containing the *STP1* promoter::*luc*+::*STP1* 3'UTR reporter construct enable the visualisation of *STP1*-driven luciferase expression; whereby the luciferase protein catalyses the emission of photons in the presence of luciferin. The photons may then be counted and imaged. The software represents increasing photon emissions with a progressive colour scale of blue, green, red, yellow, and white.

Three independent transgenic lines have been isolated, and in preliminary experiments the *STP1::luc+::3'UTR* construct appears to be regulated in a similar manner to that of *STP1* transcripts in wild-type plantlets. The greatest *STP1*-driven luciferase expression is observed in the shoots (figure 3.6.3). Furthermore, the luciferase expression is strongest in the young, expanding leaves and particularly along the mid-rib and veins of the leaves and cotyledons of transgenic plantlets. The *STP1*-driven luciferase expression can also be observed in the roots but only at very low levels (figure 3.6.4). Furthermore, it appears that *STP1*-driven luciferase expression is localised to different regions of the root in the light and dark. In the light, luciferase activity is localised predominately to the main root; whereas in the dark luciferase activity is seen in the lateral roots. However, it is difficult to be certain

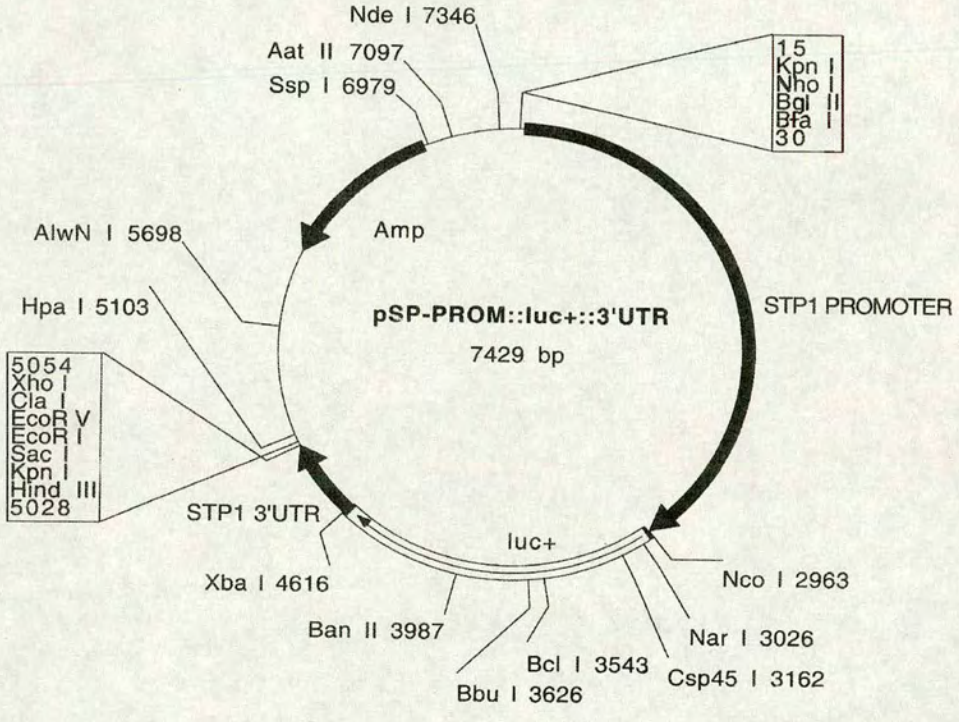


Figure 3.6.1 The diagrammatic representation of the *STP1* promoter ::*luc*+::*STP1* 3'UTR reporter construct generated in the pSP-luc+ vector.

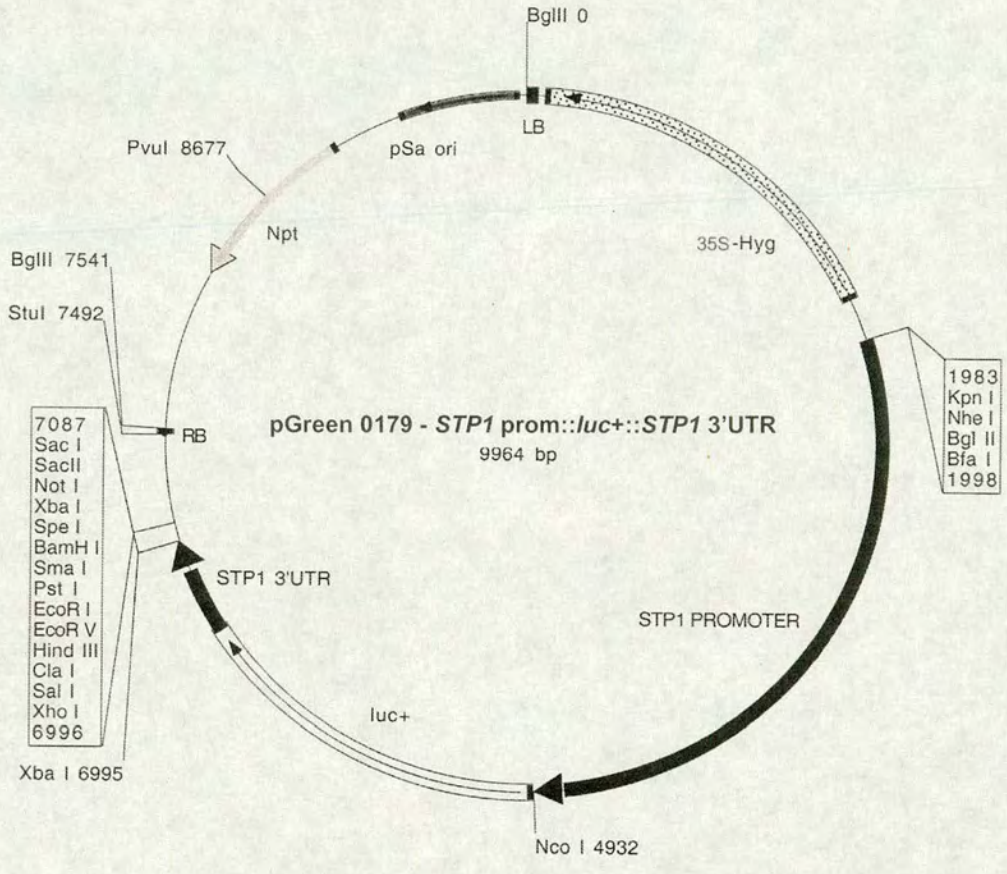


Figure 3.6.2 Diagrammatic representation of the *STP1* promoter ::luc+::*STP1* 3'UTR reporter construct in the pGreen 0179 binary vector

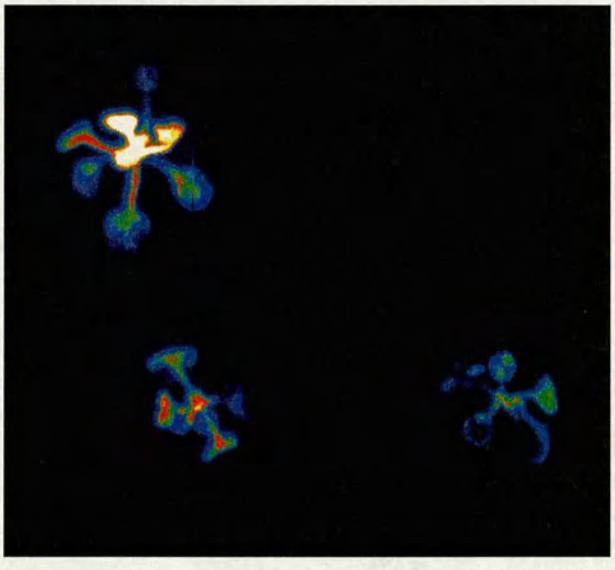


Figure 3.6.3 The imaging of the *STP1*-driven luciferase expression in the aerial parts of transgenic Col0. 8 plantlets.

Exogenous luciferin was brushed onto light-grown plantlets several hours before imaging.

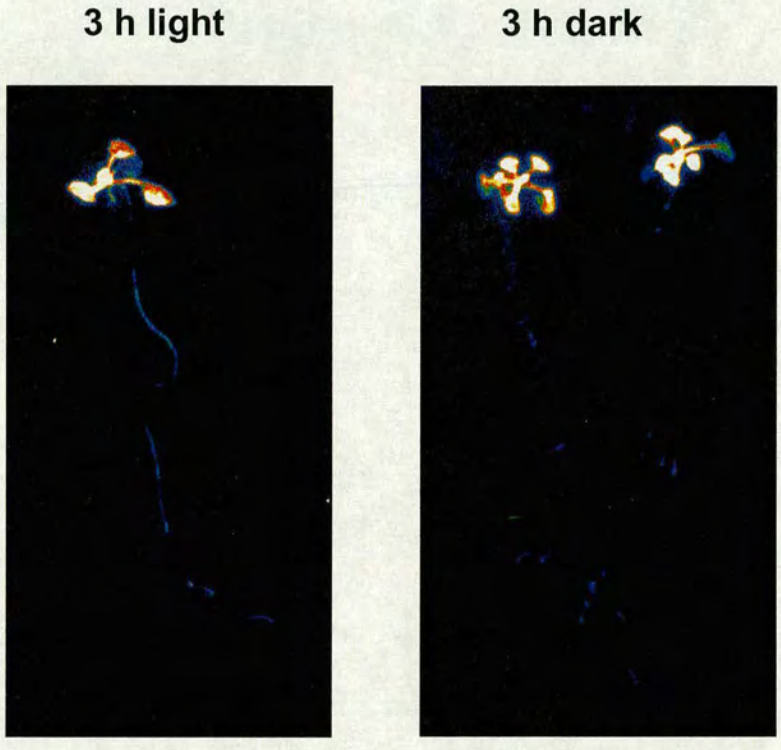


Figure 3.6.4 The horizontal view of transgenic Col0. 8 plantlets reveals *STP1*-driven luciferase expression in the roots

Exogenous luciferin was brushed onto light-grown plantlets before imaging. In the above image the camera was set to count a low number of photon emissions; therefore, regions of the plantlet with low luciferase expression were detected.

this is a real difference as the levels of photon emission are so low, and the imaging has only been done only once.

Preliminary experiments to investigate the glucose response of the *STP1:: luc::3'UTR* construct were also conducted. Light-grown transgenic plantlets were incubated in the dark for 4 h and subsequently treated with 30 mM glucose (figure 3.6.5). The *STP1*-driven luciferase expression appears to be greatest in light-grown plantlets. In fact, *STP1*-driven luciferase expression does not appear to be induced upon transfer to the dark, unlike *STP1* transcripts in Col0 wild-type plantlets (figure 3.2.1). However, *STP1*-driven luciferase expression is repressed upon incubation with 30 mM glucose.

Due to the constraints on time, equipment and plant material, the necessary controls required to make any valuable conclusions from these experiments are missing. Therefore, one can only postulate upon the reasons for differences in *STP1* gene expression and that of the *STP1* reporter construct. For example, a higher *STP1*-driven luciferase expression is seen in transgenic plantlets in the light than expected from the *STP1* transcript levels observed in wild-type plantlets. This may be due to the induction of *STP1*-driven luciferase expression as a result of mechanical stimulation from brushing the luciferin solution onto the plantlets. It is also possible that as the transgenic plantlets were not pre-treated with luciferin, any luciferase protein accumulated in the plantlet prior to the experiment may contribute to the number of photon emissions measured. Due to this factor the pre-treatment of transgenic plantlets with luciferin was performed in subsequent experiments, so only the *de novo STP1*-driven luciferase activity was measured. Alternatively, the *STP1::luc::3'UTR* construct may not be regulated in the same manner as the

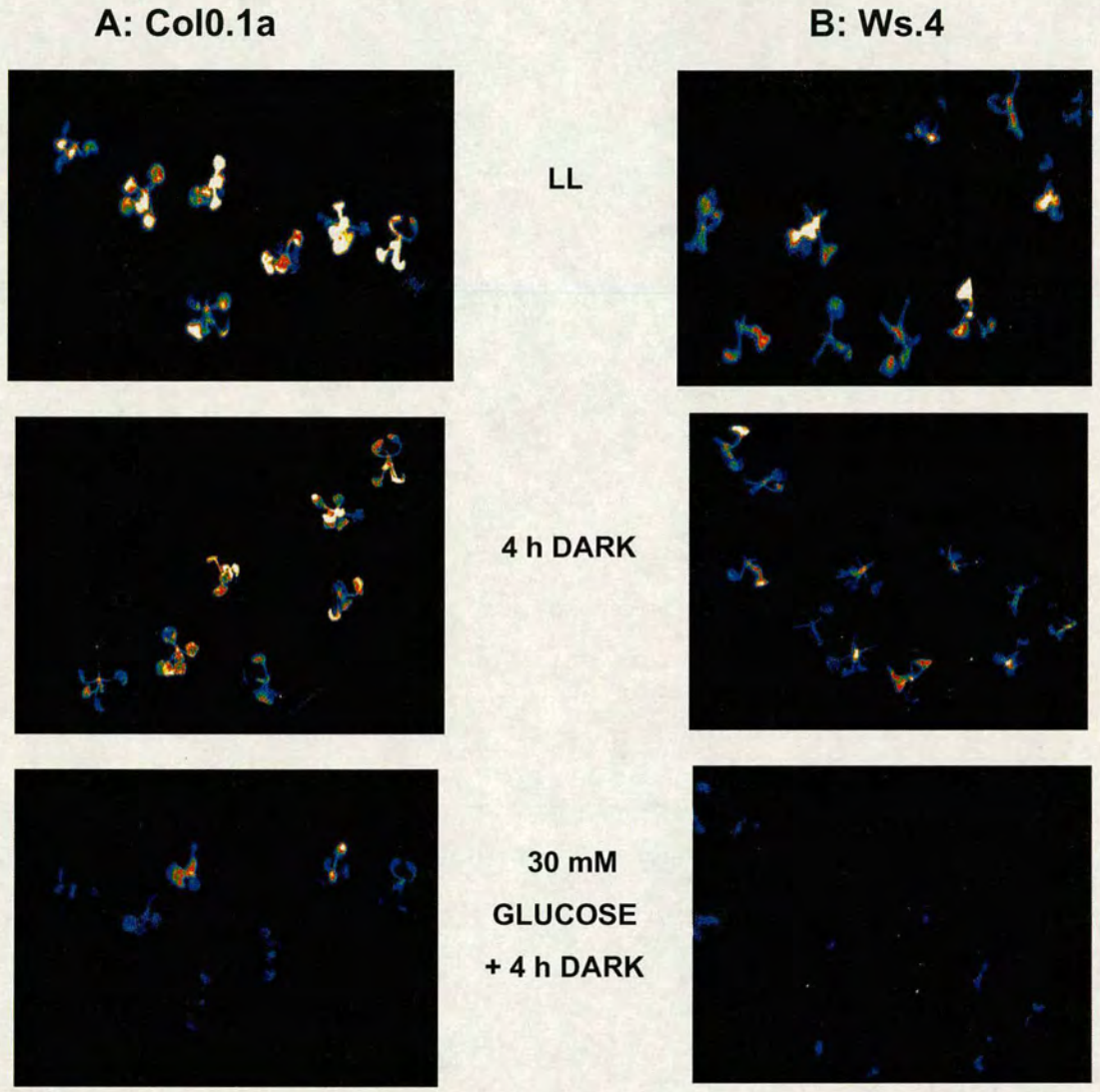


Figure 3.6.5 The response of *STP1*-driven luciferase expression to dark and glucose treatment in Col0.1a and Ws.4 transgenic plantlets.

Transgenic plantlets were grown in continuous light for 2 weeks. Plantlets were then transferred to the dark for 4 h, and subsequently treated with 30 mM glucose for a further 4 h. Plantlets were imaged at each stage of the experiment and exogenous luciferin was brushed onto plantlets before imaging.

endogenous *STP1* transcripts. If this is the case, it raises the possibility that *STP1* gene regulation is modulated either by a regulatory *cis*-element within the *STP1* coding region or by post-transcriptional modifications. More detailed experimentation is required to investigate light regulation in the *STP1::luc+::3'UTR* transgenic lines.

The *STP1*-driven luciferase expression is repressed by 30 mM glucose in all the transgenic lines (figure 3.6.5 and 3.6.6). The *STP1::luc+::3'UTR* construct appears to have a higher level of constitutive expression in the Col0.8 transgenic line relative to that in the other lines. Therefore, a more detailed study of glucose repression was performed with Col0.8 transgenic plantlets. Although, due to a limited number of plantlets available and only a 5 day trail with the imaging camera, the same transgenic plantlets were used in the experiment. Light-grown plantlets were transferred to the dark for 2 h and subsequently treated with 30 mM glucose over 6 h (figure 3.6.6). Firstly, a small increase in the *STP1*-driven luciferase expression occurs upon the transfer of plantlets to the dark. An appreciably dark-induction is not seen, the reasons for this are suggested above. Secondly, imaging of plantlets 1 h after the addition of exogenous glucose reveals a marked increase in *STP1*-driven luciferase expression. This may be attributed to mechano-stimulation, as previously demonstrated with *STP1* transcript levels (section 3.3.6). However, in plantlets incubated with 30 mM glucose for 6 h, a clear repression of *STP1*-driven luciferase expression is seen.

To investigate the glucose response of the endogenous *STP1* transcripts and *LUC* transcripts within the Col.8 transgenic line, shoot tissue was harvested from light-treated plantlets upon the subsequent incubation of plantlets in the dark with 0 mM and 30 mM glucose treatments (figure 3.6.7). The *STP1* and *LUC* transcript levels

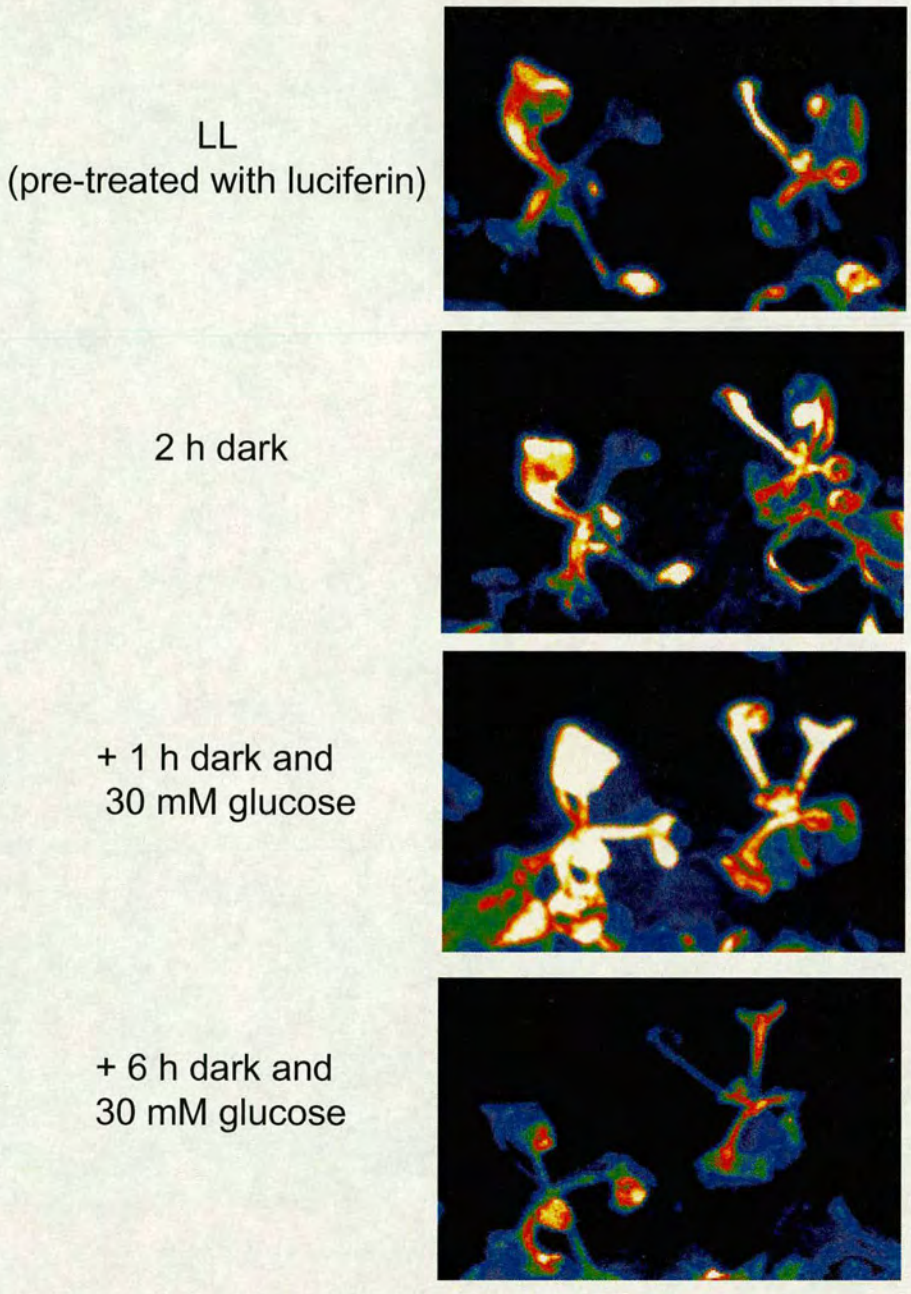


Figure 3.6.6 The response of *STP1*-driven luciferase expression to glucose in transgenic Col0. 8 plantlets

Plantlets were pre-treated with exogenous luciferin several hours before imaging of light-grown plantlets. Plantlets were transferred to the dark for 2 h and subsequently treated with 30 mM glucose for 6 h. Plantlets were imaged at each stage of the experiment.

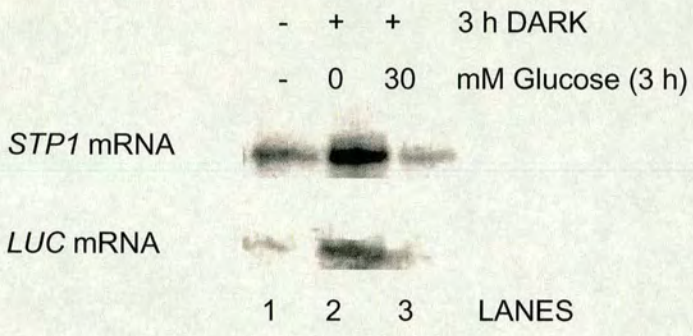


Figure 3.6.7 In Col0.8 both the *STP1* and *LUC* transcript levels respond in a similar manner to that previous reported in wild-type

Transgenic Col0 plantlets were grown for 2 weeks in continuous light. Plantlets were incubated in the dark for 3 h and subsequently treated with 0 or 30 mM glucose for a further 3 h. The expression of the *STP1* gene in the shoot tissue of treated plantlets was monitored. The RNA gel-blot was re-probed with a *LUC* specific DNA probe to reveal the *LUC* transcript levels in treated-shoot tissue.

are low in the light (lane 1). The transcript levels increase in water-treated plantlets (lane 2), but remain low in glucose-treated plantlets (lane 3). This result suggests that both the endogenous and transgenic *STP1* promoters are responsive to light and glucose treatments, in a manner similar to that of *STP1* mRNA in Col0 wild-type plantlets as described throughout this chapter.

3.7 Conclusions

The induction of *STP1* gene expression in plantlets exposed to the dark is in marked contrast to many sugar regulated genes, such as, the light inducible photosynthetic *CAB* gene (figure 3.2.1). Recently, a number of genes that are induced in dark-treated plants or in carbohydrate-starved plants have been identified, and classified as *din* genes (dark-induced) (Fujiki *et al.,* 2000 and 2001). Several *din* genes have been implicated in the savage of nutrients either during senescence or fruit-ripening.

The dark-accumulation of *STP1* transcripts seen in light-grown plantlets is unlikely to be a sole response to changing sugar levels during the light-dark transition, because a marked increase in the *STP1* transcript level occurs within 15 min of the transfer of plantlets to the dark. Therefore, it is likely that light signalling is also required to trigger the dark accumulation of *STP1* transcripts. This is consistent with experimental data indicating that *STP1* gene expression is altered in dark-incubated plantlets upon exposure to 5 min white light, in a sugar-independent manner (figure 3.4.3).

Glucose is an effective repressor of *STP1* transcripts in plantlets incubated in either the light or the dark. Furthermore, *STP1* gene expression is highly sensitive to glucose in both a concentration and time dependent manner. For example, the dark-

accumulation of *STP1* transcripts is repressed in plantlets treated with 3 mM exogenous glucose for 1 h (figure 3.3.3). Furthermore, *STP1*-driven luciferase expression is also repressed by exogenous glucose (figure 3.6.5 and 3.6.6). In the literature many genes have been described that are responsive to exogenous sugar. However, the concentration of sugar required to elicit such a response in treated plantlets, is 10 times higher than that needed to affect *STP1* gene expression (see introduction for more detail). The differences in the sugar sensitivity of such genes may be attributed to the use of different sugar response elements within the promoter or 3'UTR of the gene. Alternatively, it is possibly that the sensitivity of the *STP1* transcript glucose response is due to the additive effect of regulation both transcriptionally and post-transcriptionally. This is consistent with the rapidity of transcript turnover reported for the *Amy3D* transcript levels resulting from sugar-driven mRNA instability (Chan and Yu, 1998). Further experimentation employing transcriptional inhibitors or nuclear run-on assays to separate the regulation of *STP1* mRNA levels by transcription and mRNA stability, could be used to investigate this theory.

Therefore, the synthesis of STP1 appears to be tightly controlled by sugar. This in turn may have an impact on the partitioning of monosaccharides within *Arabidopsis* plantlets. Moreover, STP monosaccharide transport activity is also reduced by exogenous glucose (figure 3.5.1). The reduction of glucose transport activity in wild-type plantlets treated with exogenous glucose occurs in both shoot and root tissue. In fact, 30 mM glucose reduces the uptake of 3-OMG by 40% in 3 h (figure 3.5.3). Therefore, it is likely that the synthesis and the activity of the STP1 transporter protein is modulated by its transported substrate. In humans, the GLUT1 transporter may have a dual function in the transport and sensing of glucose (Bandyopadhyay *et al.,* 2000). Upon consideration of these results, it is tempting to speculate that one or more of the STPs may function in a similar manner. Another speculation is that

perhaps the STP1 transporter is auto-regulated, whereby, the activity of STP1 protein results in downstream regulation of *STP1* gene expression.

Many of the sugar-sensing mutants described in the literature, grow normally upon high sugar concentrations that impair growth and development in wild-type seedlings (detailed in section 1.7). Several such mutants are allelic to stress or phytohormone response loci. The *aba2* and *abi4* mutants are impaired in the synthesis and response of ABA respectively; they also show altered expression of some sugar-regulated genes. However, the expression of the *STP1* gene in these mutants is similar to that of wild-type, whereby the dark accumulation of *STP1* transcript levels is repressed by glucose (figure 3.3.4). Therefore, it is likely that the *STP1* gene is regulated by glucose independently of the ABA signalling pathway. It would be interesting to investigate the effect of exogenous ABA upon *STP1* gene expression and perhaps other phytohormones, such as, ethylene and brassinosteroids that have been implicated in the cross-talk between other regulatory pathways and that of sugar.

The *STP1* gene is regulated by light. Furthermore, the light response of *STP1* gene expression is rapid; an accumulation of *STP1* transcripts occurs 30 min after plantlets were transferred to the dark and subsequent exposure of dark-incubated plantlets to 5 min white light reduces the *STP1* transcript level within 1 h (Figure 3.4.3 C and A respectively). Furthermore, light and glucose appear to act independently in the repression of *STP1* transcript levels (figure 3.4.3 A).

In an attempt to identify the photoreceptor required to trigger *STP1* light response, the expression of the *STP1* gene was investigated in mutant plantlets with defective photoreceptors. The principle photoreceptors in *Arabidopsis* are phyA and phyB, which control the light signalling of many photosynthetic and *din* genes (table 1.4). However, due to functional redundancy between the phyA and phyB the light

signalling in the monogenic *phya* and *phyb* is generally not impaired. However, regulation of genes controlled by phyA and phyB is often affected in the digenic phya*/b* mutant. Nevertheless, *STP1* gene expression responds as wild-type in the above mutants (figure 3.4.4). Possible interpretations of this result are that either phyA and phyB signal transduction pathways do not regulate *STP1* gene expression, or that the experimental light condition was not stringent enough to define a specific phytochrome response (white light was used when phyA and phyB perceive R and FR respectively). Further experiments to monitor the light response of the *STP1* gene upon treatment with specific wavelengths of light and in other photoreceptor mutants, are necessary to determine the photoreceptor and signal transduction pathway required for the light regulation of *STP1* gene expression.

From the experiments in this chapter, the effect of light upon the STP 3-OMG transport activity in Col0 plantlets is unclear (section 3.5). However, the 3-OMG transport activity in the *stp1* mutant is at the least 60% lower than that in wild-type under light or dark conditions. This implies that STP1 is the major glucose transporter in *Arabidopsis* plantlets.

The in vivo localisation of the *STP1*-driven luciferase expression reveals that the initiation of STP1 synthesis occurs predominately in young expanding leaves, lateral roots and the elongation zone in the main root. This raises the possibility that the *STP1* gene is expressed in areas of cell elongation (figure 3.6.1 and 3.6.2). This result differs to the spatial distribution of STP 3-OMG transport activity in Col0 plantlets, where uptake is greatest by the roots. Differences between *STP1* transcripts and STP transport activity may be attributed to the uptake of 3-OMG by other STP transport proteins, rather than STP1. Alternatively, the synthesis and activity of the STP1 transporter may be regulated by factors downstream of mRNA, possibly by post-translational modification, or indirectly by H^+-ATPase activity.

However, as an effective STP1 protein antibody has not been successfully raised, it is not possible to monitor any changes in the STP1 protein levels. It is important that this is addressed in the future; so the possibility of post-translational regulation of the STP1 transporter can be investigated. Another possibility is that during the course of the respective experiments plantlets were subjected to differing biotic or abiotic factors, such as, mechano-stimulation

To conclude, the synthesis and activity of the STP1 transporter is regulated by light, glucose and mechano-stimulation. The expression of the STP1 gene is predominately seen in shoot tissue, particularly young expanding leaves. The possible physiological roles of the STP1 monosaccharide transporter are discussed in chapter 5.

CHAPTER FOUR: Regulation of the *STP1* gene in plantlets entrained to a diurnal light regime

4.1 Introduction

Plants are able to 'anticipate' daily changes in environmental conditions. Therefore, they are able to restrict physiological and metabolic processes to particular times throughout the 24 h day. Plants use a 24 h 'biological clock' to control the expression of genes during the normal day-night cycle (refer to section 1.10 for more details). The phase of the clock is entrained by environmental cues, such as, light-dark transitions. This provides a mechanism for detecting the length of day and night, and modulating diurnal processes through the seasonal changes in photoperiod. For example, seed germination and the shift from vegetative to floral growth can be triggered by the detection of long photoperiods/day-length (Suarez-Lopez *et al.,* 2001).

The expression of genes related to photosynthesis, starch synthesis and the utilisation of assimilates are diurnally regulated. These metabolic processes are optimised to match environmental conditions, such as photosynthesis in the light, and starch breakdown during the dark. Due to the diurnal regulation of carbon partitioning the plant is supplied with sugars throughout the day-night cycle. The distribution of sugars may also be regulated in a similar manner. This is consistent with post-translational diurnal regulation of the *Os*SUT1 and the *St*SUT1 sucrose transporters (Hirose *et al.,* 1997 and Kuhn *et al.,* 1997 respectively).

In light-grown plantlets *STP1* gene expression and transport activity is responsive to light, sugar and mechano-stimulation. To further characterise the regulation of the STP1 transporter, in this chapter the synthesis and activity of STP1 is monitored in plantlets entrained to a number of diurnal growth regimes.

The effect of exogenous glucose upon the diurnal expression of the *STP1* gene is also investigated. An alternative to using exogenous glucose treatments upon plantlets to elicit a sugar response is to use mutants that have altered sugar content and/or metabolism to wild-type. Therefore, the diurnal regulation of the *STP1* gene is also analysed in the *sex4, pgm1* and *det3* metabolic mutants. The *pgm1* mutant is deficient in the chloroplast phosphoglucomutase enzyme, resulting in a starch-deficient phenotype (Caspar et al, 1985). The *sex4* mutant is deficient in a chloroplast isoform of endoamylase, which results in a starch excess phenotype (Zeeman and Ap Rees, 1999). The *det3* mutant is deficient in the C-subunit of the V-ATPase (Schumacher *et al.,* 1999). Consequently, the mutant is impaired in its ability to load sucrose into the vacuole and sucrose accumulates in the cytoplasm of the *det3* mutant (per. comm., Dr M. Campbell, University of Oxford, UK).

In addition, the effect of light upon the expression pattern of the *STP1* gene in plantlets entrained to a diurnal (L12 h:D12 h) cycle and subsequently transferred to continuous environmental conditions is also investigated. Circadian regulation of the *STP1* gene is further analysed in the *elf3* mutant. The *elf3* mutant plant has pale leaves and elongated hypocotyls, which is a phenotype associated with defective light perception (Zagotta *et al.,* 1996). The circadian rhythm of *CAB* and *CCR2* (cold-circadian rhythm-RNA binding2) gene expression is abolished in *elf3* mutant plants in constant light (Cashmore, et al 1999). Therefore, ELF3 is postulated to function in the light input pathway of the circadian clock (Carre, 2002).

4.2 The expression of the *STP1* gene in plantlets entrained to a diurnal cycle

4.2.1 Aims

To investigate the importance of day-length upon the pattern of *STP1* gene expression, plantlets were entrained to L12 h:D12 h, L20 h:D4 h and L4 h:D20 h growth regimes and the *STP1* transcript level in the separated shoot and root tissue was monitored throughout the diurnal cycle.

The expression of the *STP1* gene was monitored in Col0 and Ws wild-type plantlets during a L12 h:D12 h regime to compare the synthesis to the activity of the STP1 transporter in both ecotypes. The rate of 3-OMG uptake by Col0, Ws and the *stp1* mutant plantlets during the diurnal cycle is addressed in section 4.3.

Arabidopsis plantlets were grown in continuous light for 8 to 10 days and subsequently entrained for at least 4 days to a diurnal growth regime prior to experimentation.

4.2.2 The expression of the *STP1* gene in plantlets entrained to a L12 h:D12 h regime

The expression of the *STP1* and *CAB* genes in Col0 plantlets

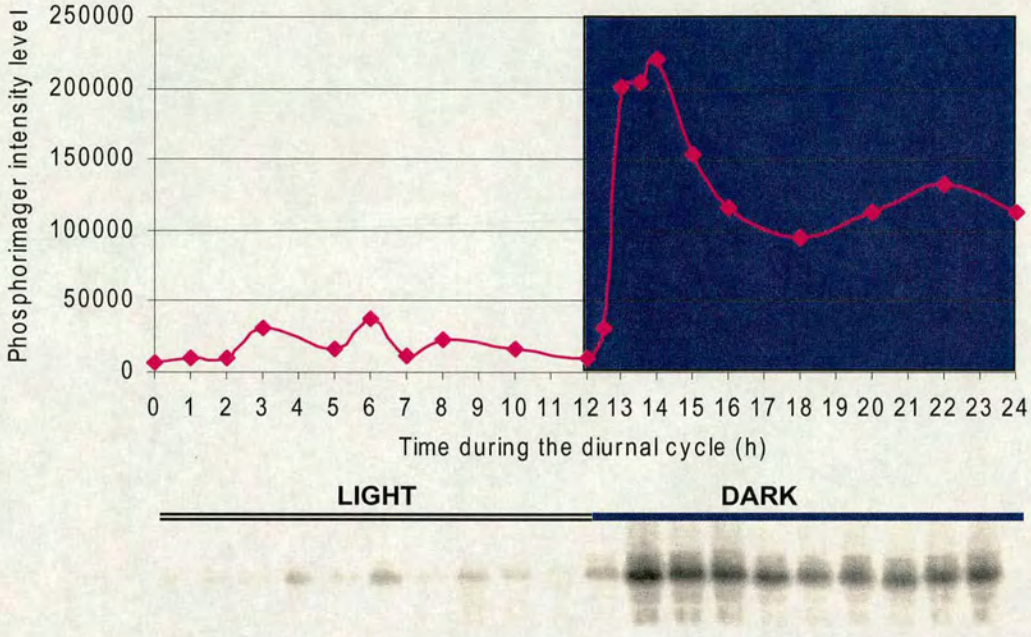
The expression of the *STP1* gene through a L12 h:D12 regime was investigated in both the shoots and the roots, following the separation of tissue upon the harvest of plantlets. Two sets of plantlets were entrained for 4 days in opposite phases every 12 h. Therefore, 0 h and 12 h samples were both harvested at the beginning of the experiment during the dark to light and light to dark transition points respectively (whilst plates were wrapped in foil upon entering the dark, or unwrapped for the light phase). The 24 h sample was collected at the end of the experiment.

The *STP1* transcript level in shoot tissue is shown on the RNA gel-blot and following quantification by phosphor-imaging (figure 4.2.1A). The expression of the *STP1* gene is not simply low in the light and high through the dark, which would be consistent with the expression of the *STP1* gene in plantlets grown solely in continuous light (figure 3.2.1). The *STP1* transcript level is low during the light phase with a small induction in the transcript level mid-phase (6 h). The *STP1* transcript accumulation after 6 h in the light was reproduced in all repeat experiments, whereas the small induction seen after 3 h light is not. The *STP1* transcript level begins to rise after entering the dark and peaks 1 h 30 min to 2 h after the light to dark transition. The transcript level remains high throughout the dark phase, with a suggestion of a second peak towards the end of the phase.

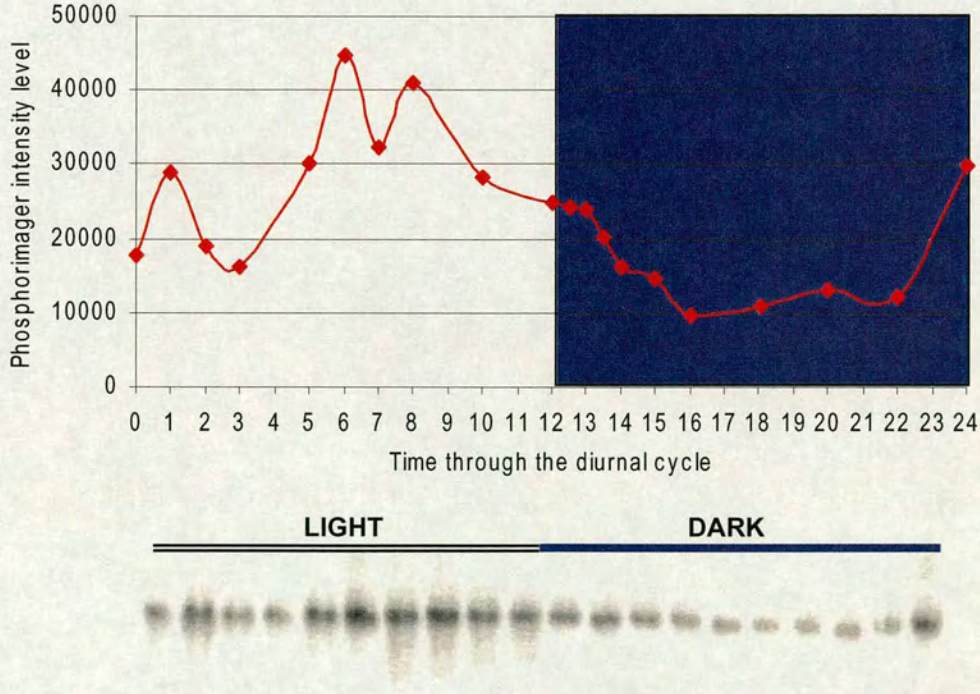
Figure 4.2.1 The expression of the *STP1* and *CAB* genes in the shoot tissue of plantlets entrained to a L12 h:D12 h regime. (next page)

Col0 plantlets were grown in continuous light for 8 days. Plantlets were then entrained to a L12 h:D12 h regime for 4 days. In this figure the mRNA from shoot tissue was monitored (figure 4.2.3 shows *STP1* transcripts in the roots). The RNA gel-blot and the quantitation of *STP1* transcripts following phosphor-imaging are shown (A). The RNA gel-blot was subsequently re-hybridised with a *CAB* specific DNA gene probe (B). Samples harvested during the light phase are represented by a white background/bar and during the dark phase by a blue background/bar.

A: *STP1* transcripts were quantified following phosphor-imaging of the RNA gel-blot



B: *CAB* transcripts were quantified following phosphor-imaging of the RNA gel-blot



The RNA gel-blot was subsequently re-hybridised with a *CAB* gene-specific DNA probe (figure 4.2.1B). The general pattern of the *CAB* gene expression observed in the shoot tissue of plantlets entrained to a L12 h:D12 h regime is the opposite to that seen with the *STP1* gene. The *CAB* transcript level begins to increase before entering the light phase and continues to accumulate to a maximum level mid-light phase (6 h). The *CAB* transcript level decreases in the latter stages of the light phase, and continues to diminish for 4 h into the dark phase. The difference in the magnitude of the transcripts between that in the light and dark phases is greater for the *STP1* gene compared to that of the *CAB* gene.

A detailed time-course to investigate the *STP1* transcript level in shoot tissue of plantlets during the light to dark phase transition is displayed in figure 4.2.2. The *STP1* transcript level begins to increase 5 min after plantlets were transferred to the dark. The transcript level continues to increase, approximately doubling every 30 min, until a maximum accumulation of *STP1* transcripts is reached 1 h 30 min after entering the dark. The *STP1* transcripts subsequently decrease to approximately half the maximum transcript level after a further 1 h 30 min in the dark.

The expression of the *STP1* gene in the roots (figure 4.2.3) displays a different pattern to that observed in the shoots of plantlets entrained to a diurnal regime (figure 4.2.1A). The expression of the *STP1* gene in root tissue during the light phase is at a comparable level to that in the shoots. A marked increase in the *STP1* transcript level is observed in the roots of plantlets, 2 h to 7 h after entering the light phase. The *STP1* transcript level increases to a maximum level 1 h after entering the dark phase. The relative accumulation is 25% of the maximum peak observed in shoots. For the remainder of the dark phase the *STP1* transcript level diminishes

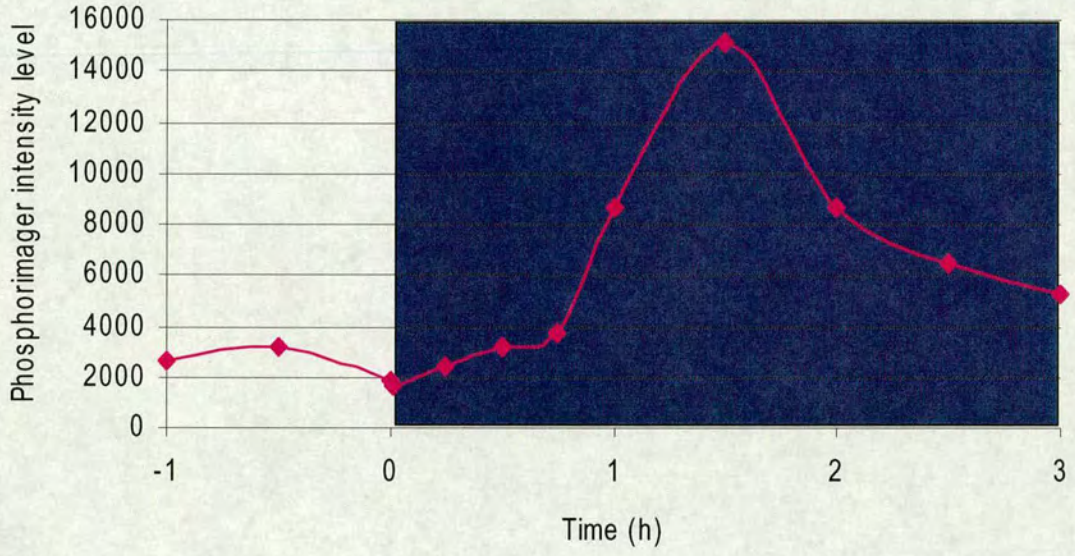


Figure 4.2.2 The expression of *STP1* gene detailed over the light-dark transition in the shoots of plantlets entrained to a L12 h:D12 h regime

Col0 plantlets were grown in continuous light for 8 days. Plantlets were then entrained to a L12 h:D12 h regime for 4 days. The *STP1* transcript level was monitored in shoot tissue during the light to dark transition. Samples harvested during the light phase are represented by a white background and during the dark phase by a blue background.

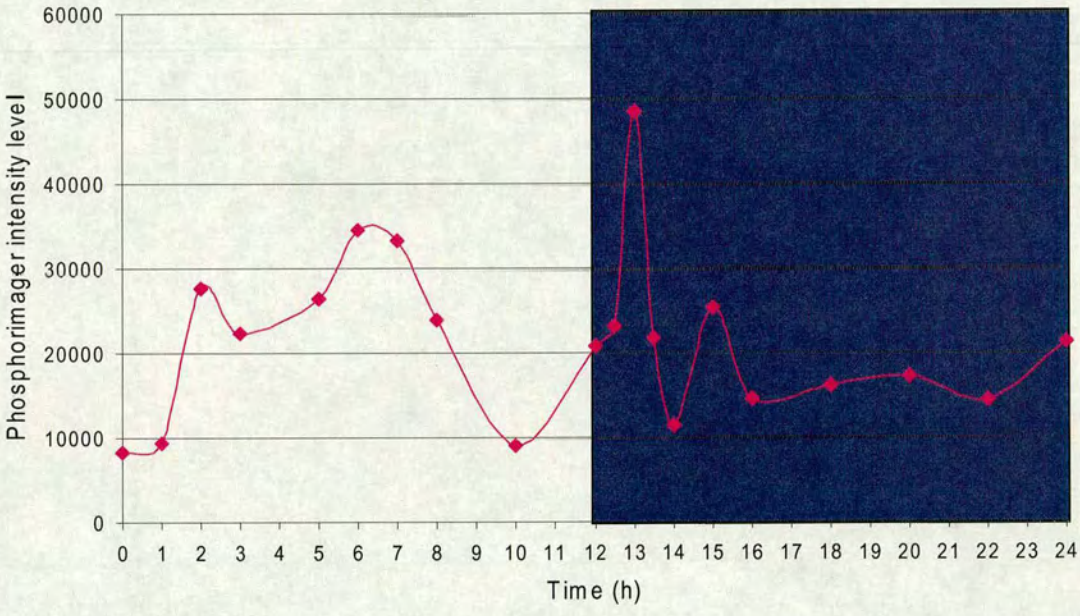


Figure 4.2.3 The expression of *STP1* gene in the roots of plantlets entrained to a L12 h:D12 h regime

Col0 plantlets were grown in continuous light for 8 days. Plantlets were then entrained to a L12 h:D12 h regime for 4 days. The *STP1* transcripts in root tissue of the plantlets were analysed (mRNA from shoots shown in figure 4.2.1). Samples harvested during the light phase are represented by a white background and during the dark phase by a blue background.

to a relatively low level, unlike the level seen in the shoots, which remains relatively high.

The expression of the *STP1* in Ws plantlets

The diurnal expression pattern of the *STP1* gene in the shoots of Ws wild-type plantlets entrained to a L12 h: D12 h regime (figure 4.2.4) is consistent with that observed in the Col0 wild-type (figure 4.2.1A). A basal level of *STP1* transcripts is observed in the shoots of plantlets during the light phase, with a small induction in the transcript amount 7 h after entering the light. Upon transfer of plantlets to the dark the shoot-specific *STP1* transcript level increases to a maximum level 2 h after the light to dark transition. A second increase in the transcript level occurs towards the end of the dark phase.

4.2.4 The expression of the *STP1* gene in Col0 plantlets entrained to a L4 h:D20 h regime

The expression of the *STP1* gene was investigated in plantlets entrained for 4 days to a L4 h:D20 h regime (figure 4.2.5), and a L20 h:D4 h (see below) to investigate the effect of day/night length upon the diurnal expression pattern of the *STP1* gene. The level of *STP1* transcripts was monitored in the shoots; therefore the results are consistent and comparable to the previous experiments where plantlets were entrained to a diurnal cycle. The 0 h sample was harvested at the beginning of the experiment ~10 min before the dark to light transition; the 4 h and 24 h samples represent light to dark and dark to light transition points respectively and the shoot

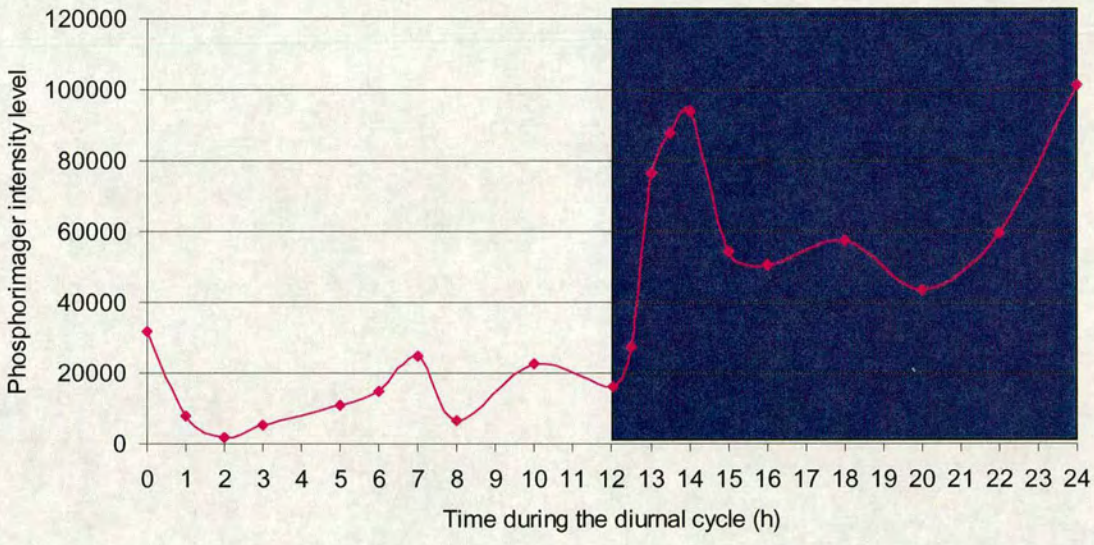
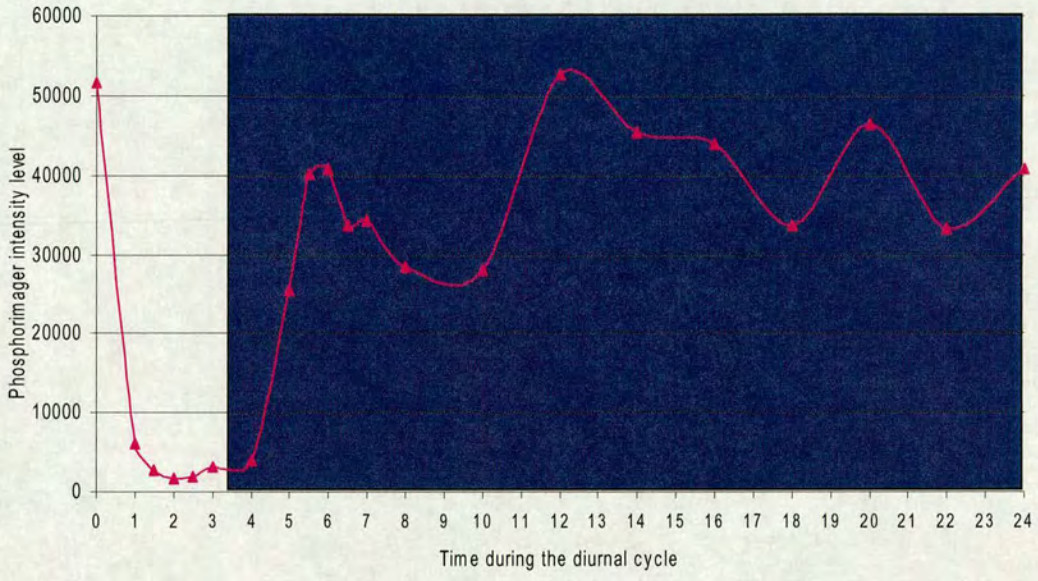


Figure 4.2.4 The expression of the *STP1* gene in the shoots of Ws plantlets upon entrainment to L12 h:D12 h regime

Ws plantlets were grown in continuous light for 8 days. Plantlets were then entrained to a L12 h:D12 h regime for 4 days. The *STP1* transcripts were quantified by phosphor-imaging of the RNA gel-blot. Shoot samples harvested during the light phase are represented by a white background and during the dark phase by a blue background.

A: *STP1* transcripts following quantitation by phosphor-imaging



B: The RNA gel-blot showing *STP1* transcripts

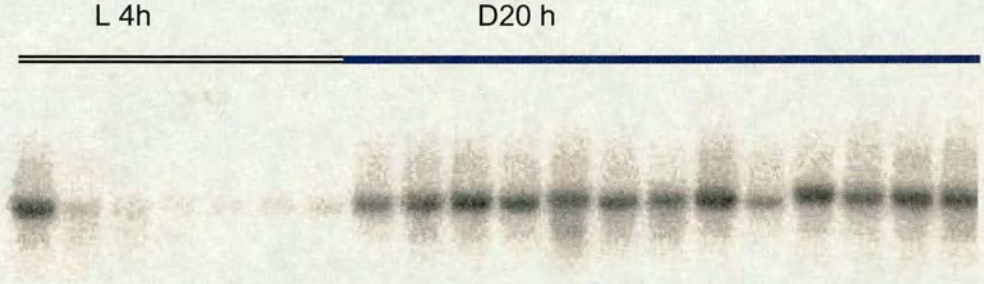


Figure 4.2.5 The expression of the *STP1* gene upon entrainment to L20 h:D4 h regime

Col0 plantlets were grown in continuous light for 8 days. Plantlets were then entrained to a L4 h:D20 h regime for 4 days. The quantitation of *STP1* transcripts represents the average intensity from phosphor-imaging of 2 gel-blots using the same RNA samples (A). One of the RNA gel-blots is also shown (B). Shoot samples harvested during the light phase are represented by a white background/bar and during the dark phase by a blue background/bar.

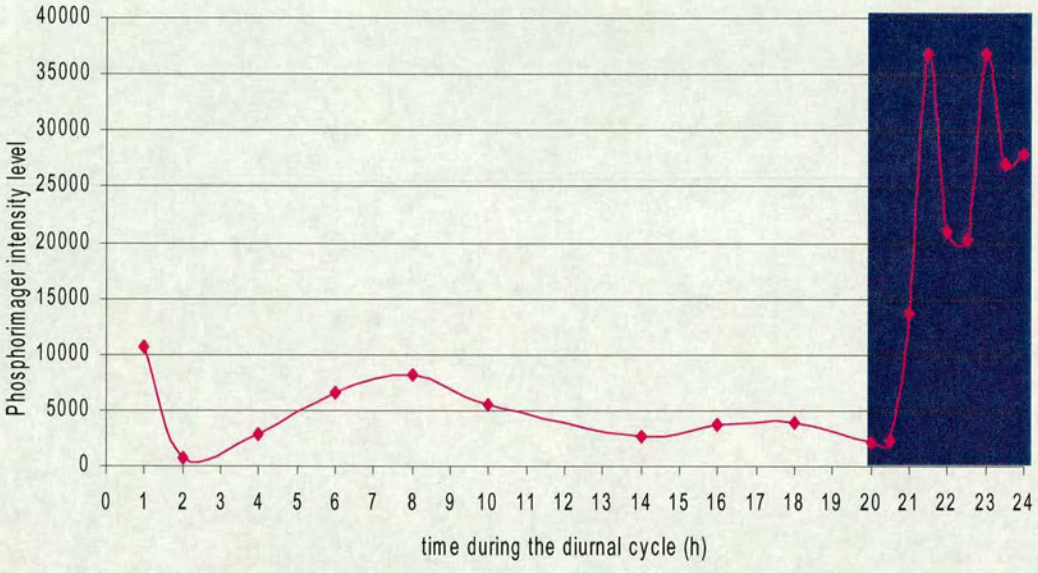
tissue was harvested during the transfer of plantlets to the next phase.

The expression of the *STP1* gene in the shoots of plantlets during a L4 h:D20 h regime was quantified by phosphor-imaging (figure 4.2.5A) of the RNA gel-blot (figure 4.2.5B). The *STP1* transcript level is high preceding the light phase (0 h) and diminishes to basal levels 1 h after entering the light. No increase in *STP1* transcripts is observed in the middle of the light phase, unlike transcripts in the shoots of plantlets entrained to a L12 h:D12 h regime (figure 4.2.1A). A peak in *STP1* transcripts occurs in the shoots of plantlets 2 h after entering the 20 h dark phase. The *STP1* transcripts accumulate to a maximum level after 12 h in the dark and remain at a relatively high level until the plantlets enter the light.

4.2.3 The expression of the *STP1* gene in Col0 plantlets entrained to a L20 h:D4 h regime

The expression of the *STP1* gene during a L20 h:D4 h regime was quantified by phosphor-imaging (figure 4.2.6 A) of the RNA gel-blot (figure 4.2.6B). The shoot tissue was harvested from plantlets during the 4 h dark phase prior to the harvest of samples from plantlets in the light phase. However, the experimental data is presented in a consistent manner with the previous diurnal cycle experiments, which detail the transcript level in the shoots of plantlets during the light phase and then in the dark phase. Therefore, no 0 h time-point is included in the figure because no shoot sample was harvested just before plantlets entered the light phase. Although, plantlets during the dark to light transition are represented by the 24 h time-point. The *STP1* transcript level is presumably high in the shoots of plantlets during the dark to light transition because the transcript level in plantlets collected at 24 h is

A: *STP1* transcripts following quantitation by phosphor-imaging



B: The RNA gel-blot showing *STP1* transcripts

Figure 4.2.6 The expression of the *STP1* gene upon entrainment to L20 h:D4 h regime

Col0 plantlets were grown in continuous light for 8 days. Plantlets were then entrained to a L20 h:D4 h regime for 4 days. The quantitation of *STP1* transcripts represents the average intensity from phosphor-imaging of 2 gel-blots using the same RNA samples (A). One of the RNA gel-blots is also shown (B). Shoot samples harvested during the light phase are represented by a white background/bar and during the dark phase by a blue background/bar.

high, and the *STP1* transcripts are still raised 1 h after entering the light, relative to that observed in plantlets during the remainder of the light phase. This suggests that the *STP1* gene does not 'anticipate' the light, but requires direct light exposure for the *STP1* transcript level to be repressed. However, as this is only one time-point, this experiment should be repeated to be certain of this.

A small peak in *STP1* transcripts occurs 6 h to 10 h after entering the light phase, which is consistent with the light peak seen in plantlets entrained to a L12 h:D12 h regime (figure 4.2.1A). Therefore, it is possible that the light phase induction of the *STP1* gene occurs after a specific number of hours (approximately 6 h) in the light rather than mid-phase. This theory is consistent with *STP1* transcript level in the shoots of plantlets entrained to a L4 h:D20 h regime, where no accumulation of *STP1* transcripts is observed during the 4 h light phase (figure 4.2.5).

Upon transfer of plantlets from the light to the dark, the shoot-specific *STP1* transcript level starts to increase 30 min after entering the dark. This is consistent with *STP1* gene expression not 'anticipating' the light to dark transition. During the 4 h dark phase a bimodal expression of the *STP1* gene is observed. An initial peak in the *STP1* transcript level occurs 1 h 30 min after plantlets enter the dark and a second peak is seen after 3 h in the dark. The latter peak of *STP1* transcripts in the shoot tissue of plantlets entrained to a L12 h:D12 h (figure 4.2.1A) or L4 h:D20 h (figure 4.2.5) regime occurs after 6 h and 10 h in the dark respectively. The expression of the *STP1* gene in the dark (discounting the initial dark-accumulation of *STP1* transcripts at 1 h 30 min) is induced at different times during dark depending on the length of the dark phase. Therefore, it is possible that the timing of the second peak of *STP1* transcripts is determined by the length of the dark phase. This may be a direct response to the length of the photoperiod or indirect via physiological factors resulting from changes in the photoperiod length.

4.3 The uptake of 3-O-methyl glucose in plantlets entrained to a L12 h:D12 h regime

4.3.1 Aims

The uptake of [^{14}C] 3-OMG by either whole plantlets or following the separation of shoot and root tissue was investigated in Col0 and Ws wild-type and *stp1* mutant plantlets entrained to L12 h:D12 h regime. The methodology of 3-OMG uptake assays is described in section 2.7.

Weighed plantlets were immersed in Gamborg medium for up to 30 min prior to the uptake assay, plantlets were assayed for 30 min and subsequently washed for 15 min; therefore plantlets may have been submerged in solution for up to 1 h 30 min prior to the extraction of radio-labelled sugars. The uptake assay was performed in laboratory light conditions. The samples for plantlets in the dark phase were covered with aluminium foil to shield them from direct light, as effectively as the uptake assay procedure allowed. The possibility that the synthesis or activity of STP1 may be altered in plantlets during the duration of the uptake experiment, relative to untreated plantlets became apparent during the course of this work and is addressed later.

4.3.2 The uptake of 3-OMG during the diurnal cycle in Col0

[^{14}C] 3-OMG uptake assays

The rate of 3-OMG uptake by whole Col0 plantlets was measured during the diurnal cycle and represents the transport activity of all the STPs (figure 4.3.1). The rate of 3-OMG uptake in plantlets during the first 6 h 30 min in the light is comparable to the uptake by plantlets grown exclusively in continuous light (figure 3.5.3). The uptake of 3-OMG by diurnally entrained plantlets begins to fall towards the end of the light phase. Upon transfer of plantlets to the dark, and during the first 3 h of the dark phase the uptake of 3-OMG by plantlets is higher than that seen in the light. As the dark phase progresses the level of 3-OMG uptake by plantlets decreases.

To further characterise the 3-OMG transport activity in Col0 plantlets during the diurnal cycle, the 3-OMG uptake assay was repeated and the shoot and root tissues were separated prior to the extraction of radio-labelled sugars (figure 4.3.2). The data-points EXP1 and EXP2 represent the results from two independent uptake assay experiments carried out on consecutive days. The uptake of 3-OMG by the shoots (figure 4.3.2A) is similar to the pattern of 3-OMG uptake by whole plantlets (figure 4.3.1). The level of 3-OMG uptake is high in shoot tissue at the beginning of the light phase, and falls as the phase progresses. Upon entering the dark phase the level of uptake by the shoots increases, peaking 4 h 30 min into the dark and subsequently falls towards the end of the phase.

The pattern of glucose transport activity observed in the roots (figure 4.3.2B) is different from that in the shoots (figure 4.3.2A). The rate of 3-OMG uptake is low at

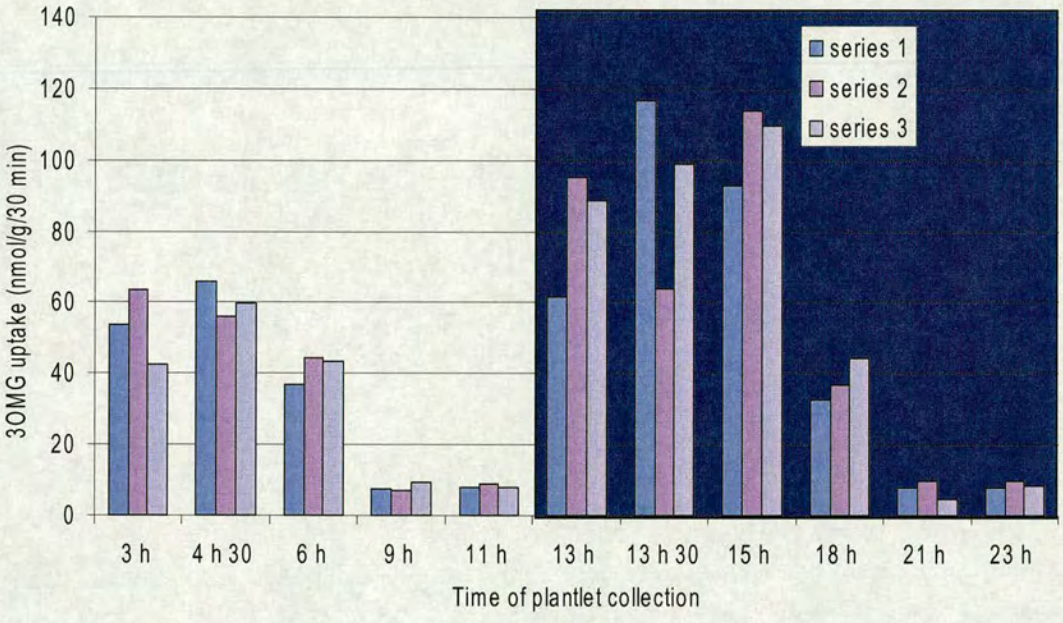


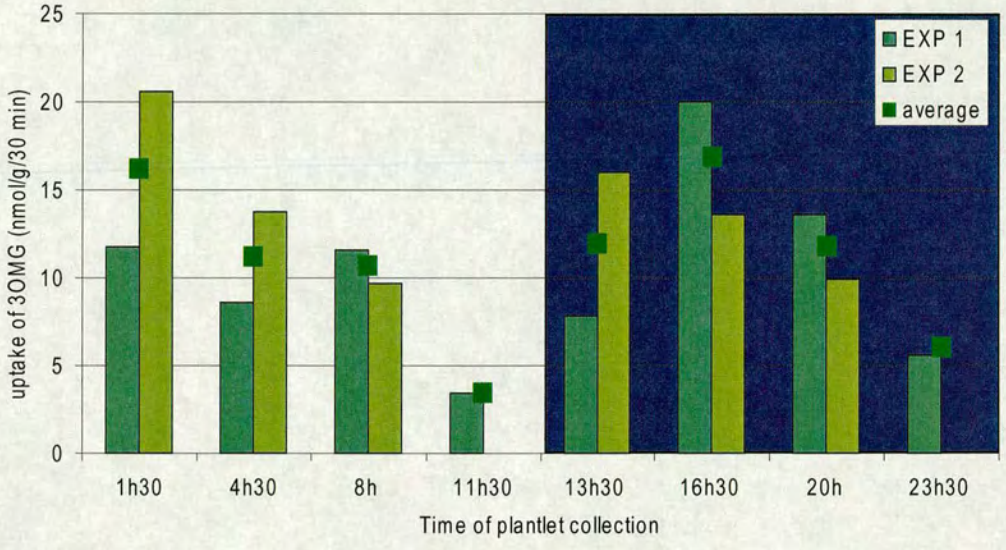
Figure 4.3.1 The uptake of [^{14}C] 3OMG in Columbia 0 plantlets upon entrainment to L12 h:D12 h

Col0 plantlets were grown in continuous light for 8 days. Plantlets were then entrained to a L12 h:D12 h regime for 4 days. The **[^{14}C]** 3OMG by the plantlets was measured throughout the diurnal cycle. Triplicate samples of plantlets were collected for each time point, which are depicted as series 1, 2 and 3 in this figure. Whole plantlet samples collected during the light phase are represented by a white background and during the dark phase by a blue background.

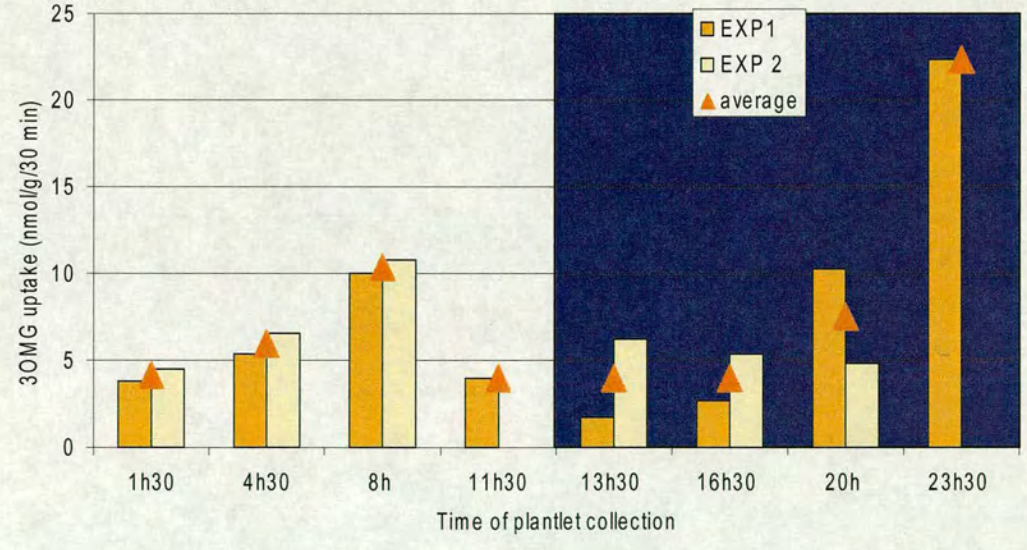
Figure 4.3.2 The uptake of [^{14}C] 3-OMG in the shoot and root tissue of Col0 plantlets during a L12 h:D12 h regime. (next page)

Col0 plantlets were grown in continuous light for 8 days. Plantlets were then entrained to a L12 h:D12 h regime for 4 days. The level of 3-OMG uptake by the plantlets was measured following the separation of shoot (A) and root (B) tissue throughout the diurnal cycle. Two independent experiments were performed on consecutive days, which are depicted as EXP1 and EXP2 in this figure. In each experiment the uptake of 3-OMG was measured in duplicate samples, the averages are shown in this figure as green boxes (uptake in shoots) and yellow triangles (uptake in roots). Samples collected during the light phase are represented by a white background and during the dark phase by a blue background.

A: The rate of 3OMG uptake in the shoots



B: The rate of 3OMG uptake in the roots



the beginning of both the light and the dark phases and increases as the phases progress peaking after 8 h in the light and 11 h 30 min in the dark. The greatest rate of 3-OMG uptake by the roots during the dark is comparable to the maximum uptake of 3-OMG observed in the shoots on a fresh weight basis.

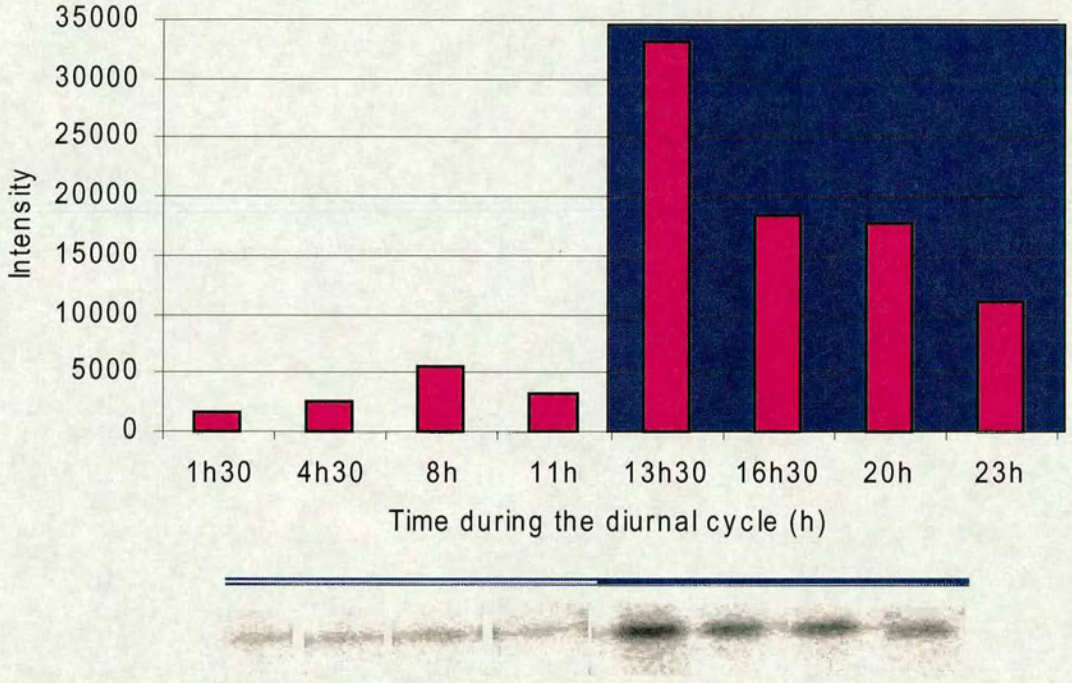
At specific times through the diurnal cycle plantlets were collected for the 3-OMG uptake assay (uptake experiment 1), at the same time plantlets were harvested to analyse the *STP1* transcript level (figure 4.3.3A). The quantification of the RNA gel-blot by phosphor-imaging reveals the *STP1* transcript level through the diurnal cycle is consistent with that shown in wild-type plantlets previously (Col0 in figure 4.2.1A and Ws in figure 4.2.4). In Col0 plantlets harvested at the beginning of the uptake experiment (figure 4.3.3A), the *STP1* transcript level is low through the light phase and elevated in the dark phase, with the highest level seen 1 h 30 min after entering the dark. The pattern of *STP1* transcripts in these experiments is different from the pattern of 3-OMG uptake in whole plantlets, shoots and roots (figures 4.3.1 and 4.3.2).

It is possible that the different pattern of *STP1* gene expression and 3-OMG transport activity through the diurnal cycle is due to changes in the synthesis or activity of STP1 during, or as a consequence of, the uptake assay. To investigate this possibility, the *STP1* transcript level was monitored in the shoot tissue of plantlets subjected to the uptake assay conditions (figure 4.3.3B). The plantlets were treated in the same way as those used in the 3-OMG uptake assay, except that the [^{14}C] 3-OMG was not added. The expression of the *STP1* gene in plantlets that were subjected to the uptake assay conditions prior to collection (figure 4.3.3B) is different

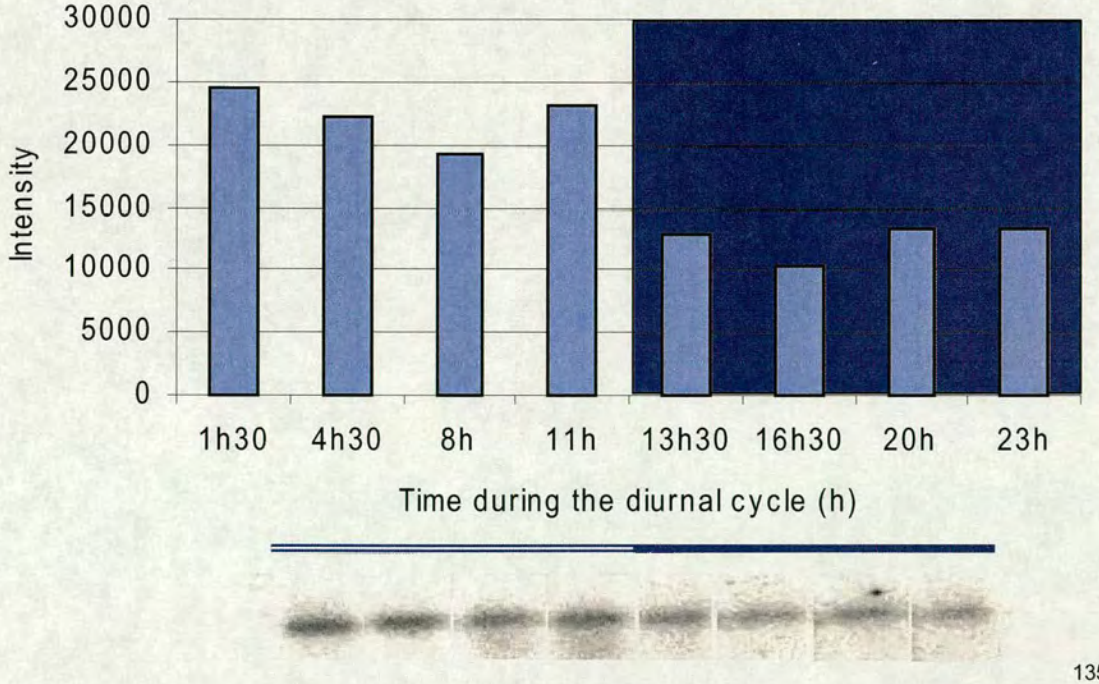
Figure 4.3.3 The expression of *STP1* gene was monitored in plantlets during the diurnal cycle, before and after plantlets were subjected to uptake assay conditions (next page)

Col0 plantlets were grown in continuous light for 8 days. Plantlets were then entrained to a L12 h:D12 h regime for 4 days. The *STP1* transcripts from plantlets harvested before (A) and after (B) the uptake assay are shown as the RNA gel-blot and following the quantification by phosphor-imaging. Samples harvested during the light phase are represented by a white background/bar and during the dark phase by a blue background/bar.

A: *STP1* transcripts in plantlets harvested before the uptake assay experiment of figure 4.3.2



B: *STP1* transcripts in plantlets harvested after being subjected to the uptake assay experiment of figure 4.3.2



from the pattern observed at the beginning of the experiment (figure 4.3.3A). In shoot tissue harvested following the uptake assay, the *STP1* transcript level during the light phase is 5 times higher than that at the beginning of the experiment and no peak in *STP1* transcripts is seen after 1 h 30 min in the dark. In general the *STP1* transcript level in the shoots of plantlets subjected to the uptake assay is at a lower level throughout the dark phase, relative to that in plantlets harvested before the assay. The rapid changes in *STP1* gene expression during the uptake assay may be due to the effects of mechano-stimulation and/or light, resulting in an induction of light phase *STP1* transcripts and a decrease in dark phase *STP1* transcripts respectively. Therefore, it is likely that such rapid changes in the regulation of the *STP1* gene, and possibly that of other *STP* genes, contribute to the differences between the levels of *STP1* transcripts (figure 4.3.3A) and 3-OMG uptake (figure 4.3.1 and 4.3.2) in the shoots and roots of plantlets entrained to a L12 h:D12 h regime.

Autoradiography of plantlets following the uptake of 3-OMG

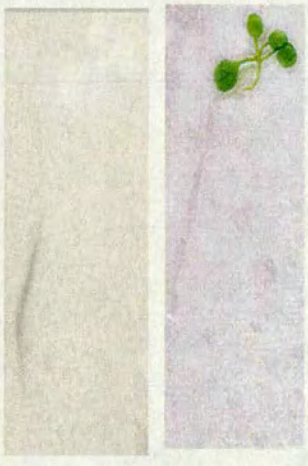
To investigate the localisation of 3-OMG uptake by the STPs in wild-type plantlets at specific times during the diurnal cycle, X-ray films were exposed to plantlets following the [^{14}C] 3-OMG uptake assay (figure 4.3.4). The autoradiography of plantlets through a L12 h:D12 h regime reveals that after 2 h in the light phase the uptake of 3-OMG is only observed in the roots and as the phase progresses the uptake of 3-OMG increases in the shoots. After 4 h 30 min in the dark the rate of uptake is high in both the shoot and root tissue. As the dark phase progresses the uptake of 3-OMG in the shoot decreases but that in the roots remains high. The

Figure 4.3.4 The uptake of [^{14}C] 3-0-methyl glucose by Col0 plantlets during a L12 h:D12 h regime. (next page)

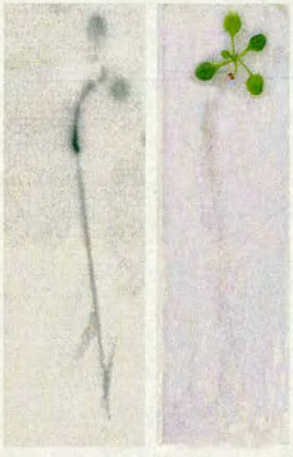
Col0 plantlets were grown in continuous light for 8 days. Plantlets were then entrained to a L12 h:D12 h regime for 4 days. Samples collected during the light phase are represented by a white bar and during the dark phase by a blue bar. The distribution of 3-OMG in plantlets was determined qualitatively, following the exposure of x-ray films to the assayed plantlets for 1 month. At each time point, the left-hand image depicts 3-OMG uptake by plantlets, and the right-hand image is a photograph of the plantlet.

LIGHT

2 h



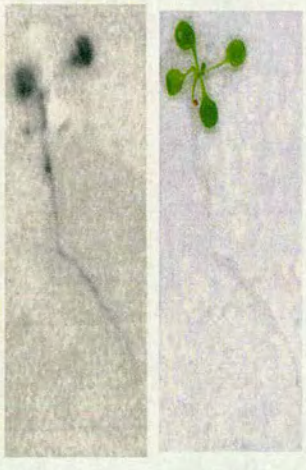
4 h



10 h

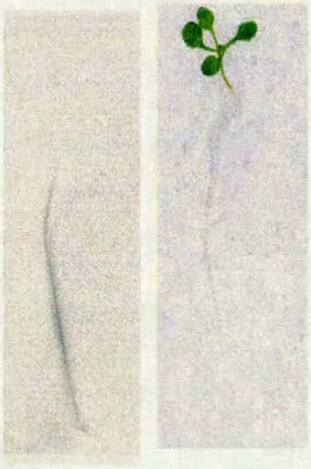
DARK

16 h 30 min



19 h

22 h 15 min



pattern of 3-OMG distribution through the L12 h:D12 h cycle is not consistent with the quantification of 3-OMG distribution in the shoots and roots (figure 4.3.2). The differences could possibly be attributed to the fact that plantlets were collected at different times for the autoradiography experiment and the uptake assays, or because the uptake of 3-OMG for autoradiography was assayed for 15 min rather than 30 min for the uptake assay, or perhaps the plantlets in each experiment were subjected to mechano-stimulation and light to different extents. Overall these observations indicate that *STP1* gene expression and STP activity may be highly responsive to abiotic factors and that their responses may differ in the shoot and root.

4.3.3 The rate of 3-OMG uptake in wild-type and *stp1* mutant plantlets

The uptake of 3-OMG in Ws through the L12 h:D12 h cycle was investigated so direct comparisons could be made between wild-type and the *stp1* mutant (Ws ecotype) (figure 4.3.5). In general, the pattern of 3-OMG uptake through a diurnal cycle by Ws plantlets is similar to that by Col0. The level of 3-OMG uptake by plantlets entering the light phase increases, peaking 6 h into the light and subsequently falls towards the end of the phase. The uptake of 3-OMG by plantlets in the dark phase is also high, reaching a maximum 6 h after entering the dark. The uptake of 3-OMG by *stp1* mutant plantlets remains at a consistently low level throughout the diurnal cycle. The amount of 3-OMG taken up by *stp1* mutant plantlets is more than 60% lower than that by wild-type plantlets, with the exception of the 9 h light time-point where the uptake of 3-OMG is the same in both Ws and *stp1* mutant plantlets. This is consistent with previous reports that STP1 is the major glucose transporter in *Arabidopsis* plantlets (chapter 3.5; Sherson *et al.,* 2000)

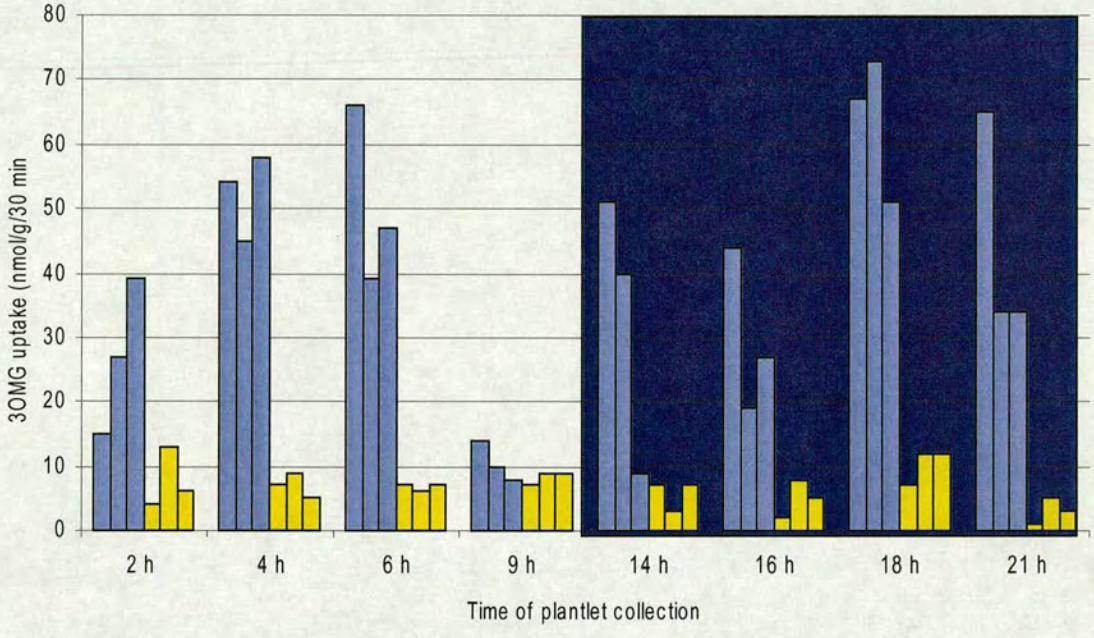


Figure 4.3.5 The uptake of [^{14}C]3-O-methyl glucose by Ws and *stp1* mutant plantlets during a L12 h:D12 h regime

Ws and *stp1* mutant plantlets were grown in continuous light for 8 days. Plantlets were then entrained to a L12 h:D12 h regime for 4 days. The 3OMG uptake by the plantlets was measured throughout the diurnal cycle. Triplicate samples of plantlets were collected for each time point, which are represented by blue bars for Ws and yellow bars for *stp1* mutant. Samples collected during the light phase are represented by a white background and during the dark phase by a blue background.

Furthermore, these results suggest the diurnal pattern of 3-OMG uptake is principally due to STP1 transport activity rather than other STPs, in plantlets entrained to L12 h:D12 h regime.

4.4 The light response of *STP1* gene expression in plantlets entrained to a L12 h:D12 h regime

4.4.1 Aims

Arabidopsis plantlets were entrained to a L12 h:D12 h regime, and subsequently transferred to continuous light (LL) or dark (DD) for 48 h to investigate the circadian regulation of *STP1* gene expression. The Col0 plantlets used were either grown in continuous light and entrained to a L12 h:D12 h regime for 4 days by wrapping plates in foil (as used in all experiments reported in this chapter so far); or plantlets were grown in a growth room set to a L12 h:D12 h regime. Plantlets grown in the growth room may have been exposed to low levels of light during the dark phase due to light leaking into the growth room, e.g., under the door, through ventilation ducts etc. The potential importance of this was only realised during the course of this work.

In addition, the circadian expression of the *STP1* gene in *elf3* arrhythmia mutant plantlets was monitored. The ELF3 receptor is thought to function in maintaining the light input to the "biological clock"; and consequently environmental signals can re-set the clock. In the absence of ELF3 the expression of several clock-controlled genes, such as *CAB* and *CCR2,* are arrhythmic.

4.4.2 The expression of the *STP1* gene upon transfer to LL

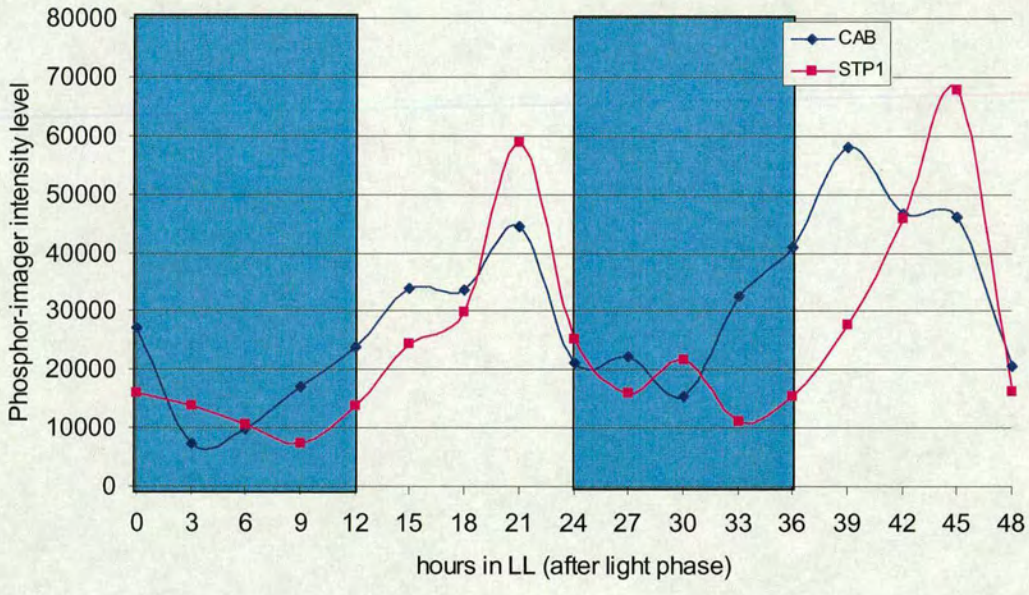
Plantlets grown in L12 h:D12 h regime or entrained to L12 h:D12 h for 4 days display very similar expression patterns of the *STP1* gene upon transfer to LL (only the latter is shown below). The shoot and root tissues were separately and subsequently harvested during the 48 h LL treatment so that the circadian rhythm of the *STP1* gene could be investigated in both tissues. Furthermore, the *STP1* transcript levels in the shoot tissue of plantlets entrained to a L12 h:D12 h regime (figure 4.2.1A), and those in plantlets subsequently transferred to LL (figure 4.4.1), or DD (figure 4.4.3) are comparable. This is because firstly, the experiments were performed on the same batch of plantlets that were treated in the same way. Secondly, the RNA samples from all the experiments were equally loaded relative to each other. Thirdly, the RNA from the 3 experiments was transferred to the same membrane; therefore the *STP1* transcripts were quantified on the same RNA gel-blot following hybridisation using the same DNA probe. The same applies to *CAB* gene expression displayed in the figures.

Plantlets were entrained in a L12 h:D12 h regime for 4 days and at the end of the light phase were transferred to LL for 48 h. The 0-12 h and 24-36 h time-points represent subjective night (shaded background) and 12-24 h and 36-48 h time-points subjective day (white background). The expression of the *STP1* gene in the shoots of plantlets transferred to LL was quantified by phosphor-imaging (figure 4.4.1A) of the RNA gel-blot (figure 4.4.1B). The *STP1* transcript level is comparable to that observed in the shoots of plantlets during the light phase of L12 h:D12 h cycle (figure 4.2.1A). The maximum level of shoot-specific *STP1* transcripts in the plantlets transferred to LL is marginally higher than that observed in plantlets entrained to a L12 h:D12 h regime after 6 h in the light (figure 4.2.1A). Two peaks in

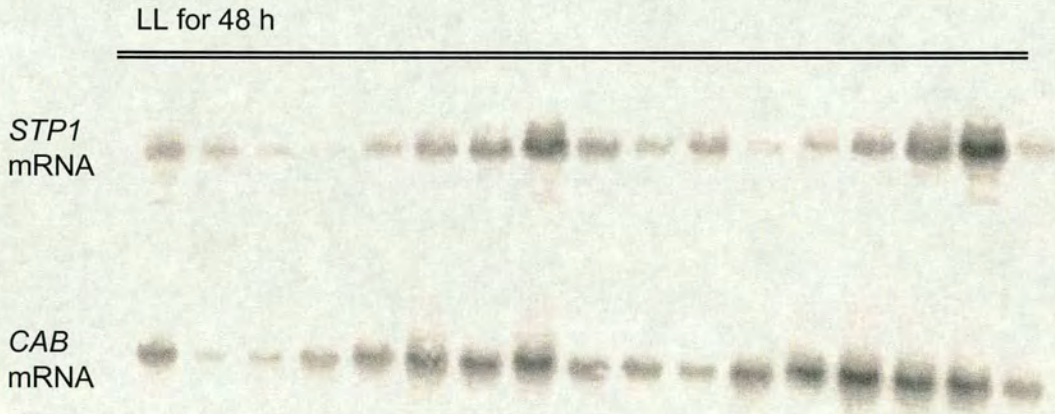
Figure 4.4.1 The expression of the *STP1* and *CAB* genes in the shoots of plantlets transferred to continuous light following a 4 day entrainment to a L12 h:D12 h regime. (next page)

Col0 plantlets grown in continuous light for 10 days were entrained to a L12 h:D12 h regime. Plantlets were subsequently transferred to LL for 48 h and tissue samples were harvested every 3 h. Upon harvest the shoot and root tissue was separated (mRNA of the root tissue is displayed in figure 4.4.2). The *STP1* and *CAB* transcripts were quantified by phosphor-imaging (A) of the RNA gel-blot (B). Samples harvested during the subjective day are represented by a white background and during the subjective night by a blue background.

A: *STP1* and *CAB* transcripts in the shoots were quantified by phosphor-imaging



B: The RNA gel-blot of *STP1* and *CAB* transcripts in the shoots



the *STP1* transcript level are observed in the shoots, 21 h and 45 h after plantlets were transfer to LL. Furthermore, the peaks in *STP1* transcripts have a periodicity of 24 h and occur 9 h into the subjective day. Therefore, *STP1* gene expression in the shoots displays a circadian rhythm in LL that may correspond to the induction of *STP1* transcripts mid-phase in plantlets entrained to a diurnal cycle. No increase in *STP1* gene expression is apparent during the subjective night, which is unexpected because in plantlets entrained to a L12 h:D12 h regime the *STP1* transcripts accumulate to a maximum level in the dark phase. The expression of the *STP1* gene is comparable to that of the *CAB* gene in magnitude and timing during the 48 h LL (figure 4.4.1). Even though, in plantlets entrained to a diurnal cycle the peak in *CAB* transcripts is seen during the light phase, whereas *STP1* transcripts peak in the dark phase (figure 4.2.1B and A respectively).

The *STP1* transcript level in the roots of plantlets transferred to LL for 48 h is low relative to level in the shoots (figure 4.4.2). Furthermore, no clear circadian rhythm is apparent.

4.4.3 The expression of the *STP1* gene upon transfer to DD

The expression pattern of the *STP1* gene in the shoots of plantlets transferred to DD differs depending on whether plantlets are grown in a growth room set to a L12 h:D12 h regime or entrained for 4 days to a L12 h:D12 h regime. The circadian expression of the *STP1* gene in the shoots of plantlets entrained in a L12 h:D12 h regime for 4 days is addressed in figure 4.4.3. The plates of Col0 plantlets were grown in continuous light for 10 days, and were subsequently entrained to a diurnal cycle by wrapping the plates in foil during the dark phase. At the end of the fourth

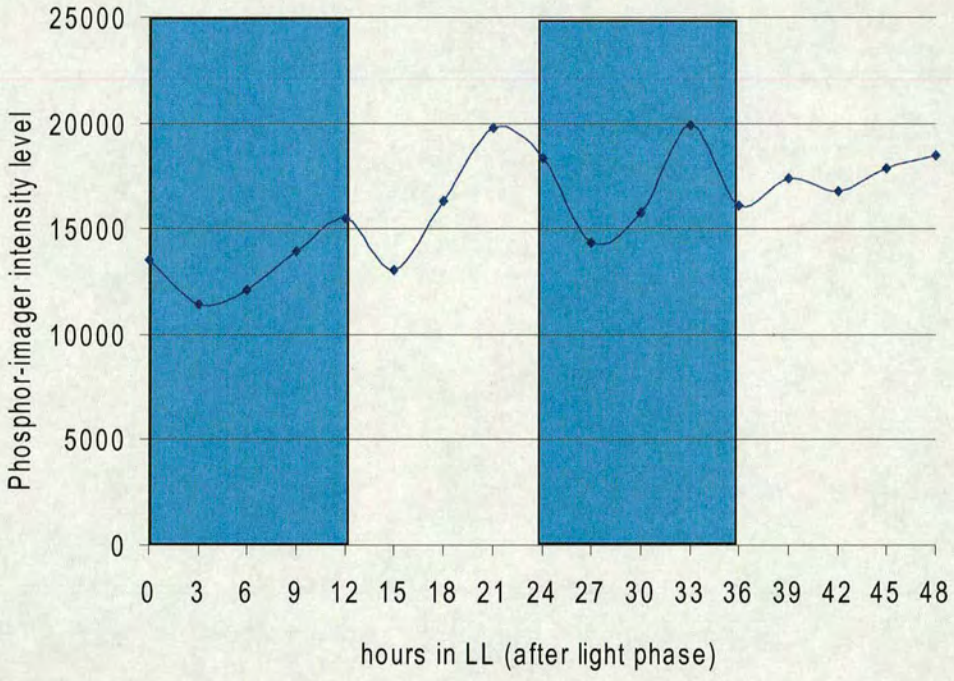


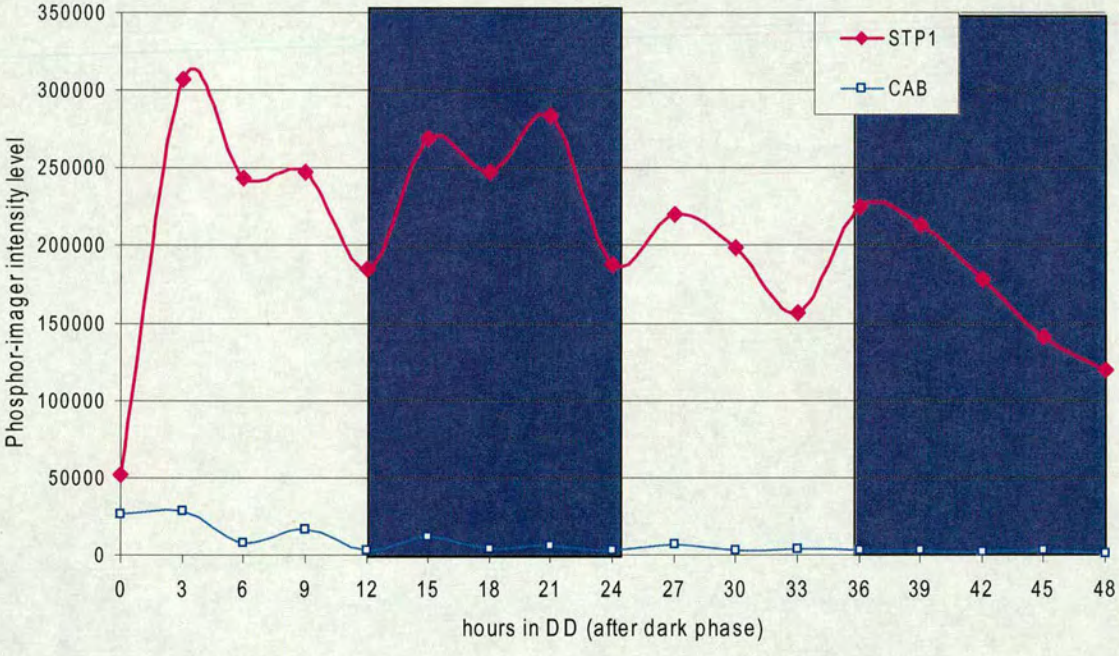
Figure 4.4.2 The expression of the *STP1* gene in the roots of plantlets transferred to continuous light following entrainment to a L12 h:D12 h regime

Col0 plantlets grown in continuous light for 10 days were entrained to a L12 h :D12 h regime. Plantlets were subsequently transferred to LL for 48 h and tissue samples were harvested every 3 h. Upon harvest the shoot and root tissues were separated (mRNA of the shoot tissue is displayed in figure 4.4.1). The *STP1* transcripts are displayed upon quantitation of the RNA gel-blot. Samples harvested during the subjective day are represented by a white background and during the subjective night by a blue background.

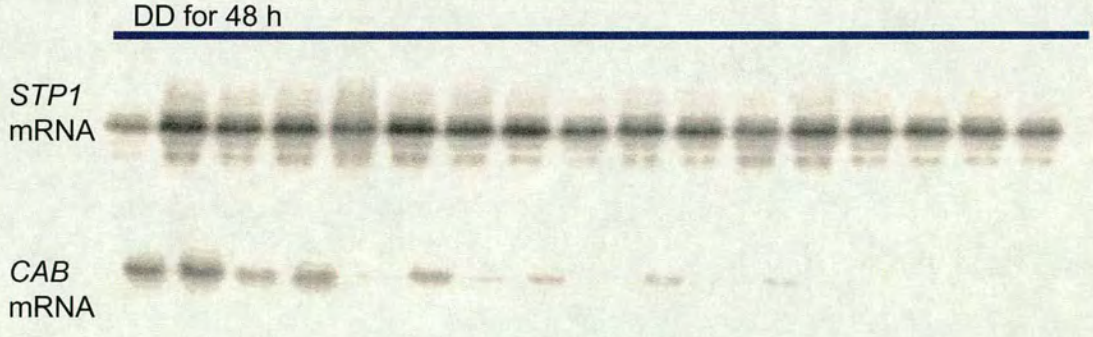
Figure 4.4.3 The expression of the *STP1* gene in the shoots of plantlets transferred to continuous dark following a 4 day entrainment to a L12 h:D12 h regime

Col0 plantlets grown in continuous light for 10 days were entrained to a L12 h:D12 h regime. Plantlets were subsequently transferred to DD for 48 h and tissue samples were harvested every 3 h. Upon harvest the shoot and root tissue was separated (mRNA of the root tissue is displayed in figure 4.4.4). The *STP1* and *CAB* transcripts were quantified by phosphor-imaging (A) of the RNA gel-blot (B). Samples harvested during the subjective day are represented by a white background and during the subjective night by a blue background.

A: *STP1* and *CAB* transcripts in the shoots were quantified by phosphor-imaging



B: The RNA gel-blot of *STP1* and *CAB* transcripts in the shoots



dark phase the plantlets were transferred to DD for 48 h; therefore 0-12 h and 24-36 h represent subjective day (white background) and 12-24 h and 36-48 h subjective night (shaded background). The expression of the *STP1* gene in the shoots upon transfer to DD was quantified by phosphor-imaging (figure 4.4.3A) of the RNA gel-blot (figure 4.4.3B). No apparent circadian rhythm in the *STP1* transcript level is seen, but the level remains high throughout the 48 h DD. The transcript level is comparable to that observed in the shoots during the dark phase, in plantlets entrained to a L12 h:D12 h regime (figure 4.2.1A). The expression of the *CAB* gene upon transfer of plantlets to DD is very low compared to that of the *STP1* gene and no circadian rhythm is apparent.

The expression of the *STP1* gene in the roots of plantlets transferred to DD (figure 4.4.4) is at an equivalent level to that seen in the roots of plantlets transferred to LL (figure 4.4.2). No apparent circadian rhythm is observed, although the expression pattern in the roots of plantlets after the first 12 h DD resembles that seen in the shoots with a delay of 6 h (figure 4.4.3). This experiment would need to be repeated before any significant meaning could be drawn from the data.

The circadian regulation of the *STP1* gene was also investigated in plantlets previously grown for 3 weeks in a growth room set to a L12 h:D12 h regime (figure 4.4.5). At the end of the dark phase, plantlets were transferred to DD for 48 h. The *STP1* transcript level in the shoots was quantified by phosphor-imaging (figure 4.4.5A) of the RNA gel-blot (figure 4.4.5B). Two peaks in the *STP1* transcript level occur in the shoots of plantlets at 18 h and 39 h in DD; which correspond to 6 h and 3 h into the subjective night respectively. The expression of the *STP1* gene in DD displays a circadian rhythm that may correspond to the dark accumulation of *STP1*

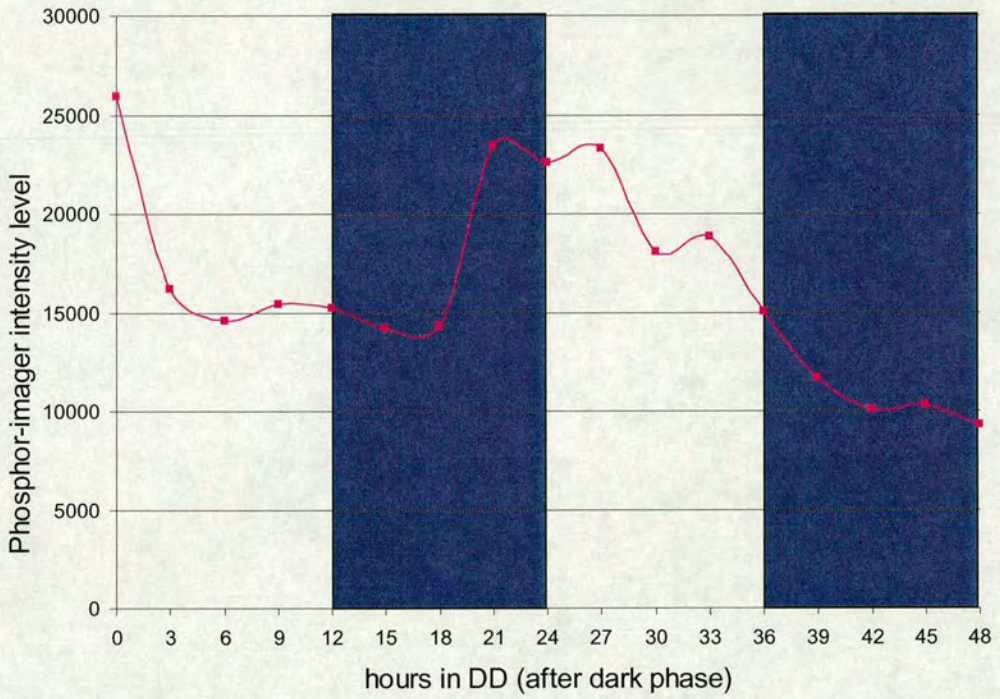
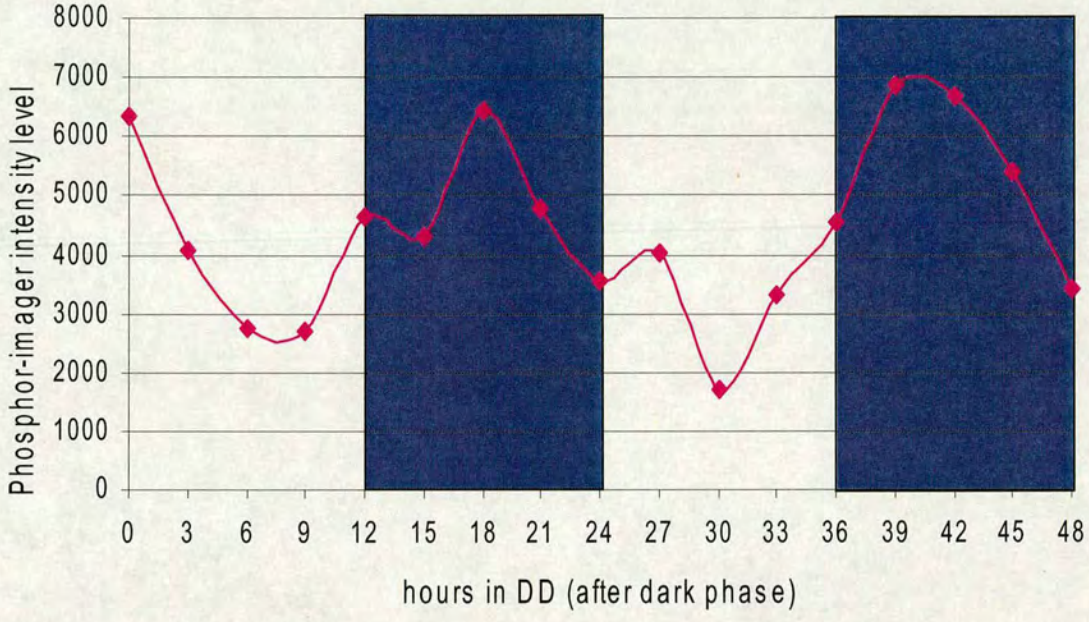


Figure 4.4.4 The expression of the *STP1* gene in the roots of plantlets transferred to continuous dark following entrainment to a L12 h:D12 h regime

Col0 plantlets grown in continuous light for 10 days were entrained to a L12 h:D12 h regime. Plantlets were subsequently transferred to DD for 48 h and tissue samples were harvested every 3 h. Upon harvest the shoot and root tissues were separated (mRNA of the shoot tissue is displayed in figure 4.4.3). The *STP1* transcripts are displayed upon quantitation of the RNA gel-blot. Samples harvested during the subjective day are represented by a white background and during the subjective night by a blue background.

A: *STP1* transcripts in the shoots were quantified by phosphor-imaging



B: The RNA gel-blot of *STP1* transcripts in the shoots

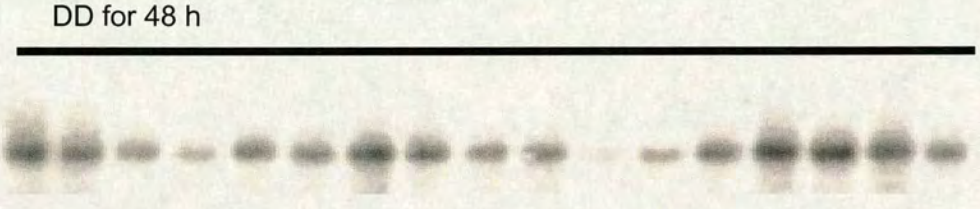


Figure 4.4.5 The expression of the *STP1* gene in plantlets transferred to continuous dark following growth in a L12 h:D12 h regime for 3 weeks

Col0 plantlets were grown in a L12 h:D12 h regime for 3 weeks. Plantlets were subsequently transferred to DD for 48 h and tissue samples were harvested every 3 h. Upon harvest the shoot and root tissues were separated (mRNA of the root tissue is displayed in figure 4.4.6) . The *STP1* transcripts were quantified by phosphor-imaging (A) of the RNA gel-blot (B). Samples harvested during the subjective day are represented by a white background and during the subjective night by a blue background.

transcripts observed in plantlets entrained to a L12 h:D12 h regime during the dark phase (figure 4.2.1A).

In plantlets transferred to DD following a 4 day entrainment to a L12 h:D12 h regime no clear circadian rhythm is observed. Whereas, in plantlets grown in a growth room set to a L12 h:D12 h regime a circadian peak in *STP1* transcript is seen during the subjective night. The differences in the circadian expression of the *STP1* gene of these plantlets may be because 4 days is not sufficient to entrain plantlets to a diurnal cycle, or possibly because wrapping the plates in aluminium foil results in a strong dark induction of *STP1* transcripts that masks any potential rhythm.

Furthermore, upon transfer of plantlets grown in growth room in a diurnal cycle for 3 weeks to DD for 48 h, the circadian expression of the *STP1* gene in the roots was monitored (figure 4.4.6). The *STP1* transcript level peaks during each 12 h period, which corresponds to both subjective day and night. It is possible that these peaks in *STP1* gene expression correspond to the light and the dark phase peaks observed in the diurnal cycle (Fig 4.2.3). In fact, the pattern of expression is the same as that seen in the roots of plantlets grown for 3 weeks in a L12 h:D12 h (not shown; figure 4.2.3 represents mRNA from roots of plantlets entrained for 4 days). Therefore, in the roots the *STP1* gene expression displays a rhythm with a periodicity of 12 h with a similar magnitude in both the subjective day and night.

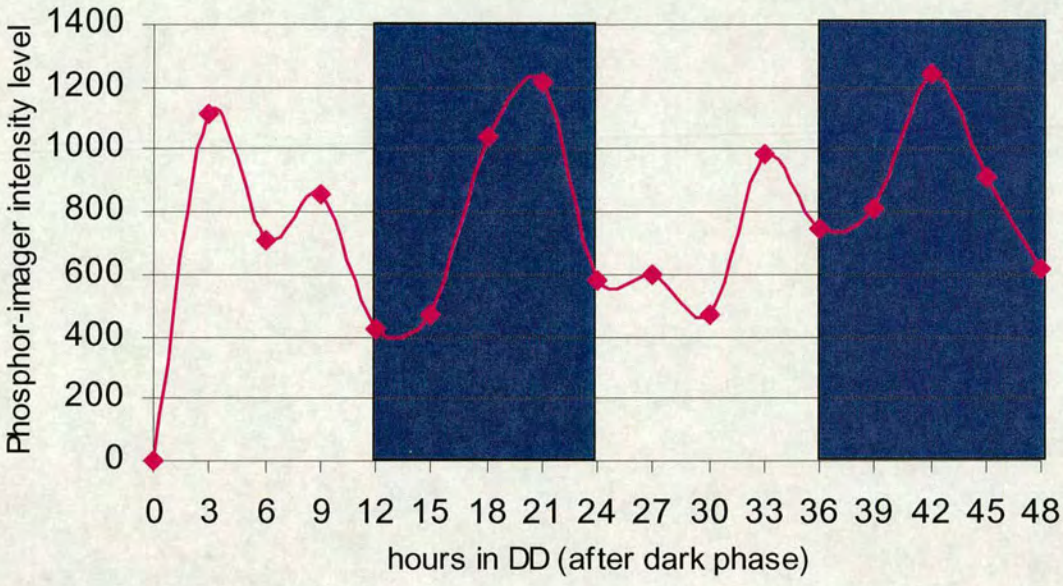


Figure 4.4.6 The expression of the *STP1* gene in the roots of plantlets transferred to continuous dark following entrainment to a L12 h:D12 h regime

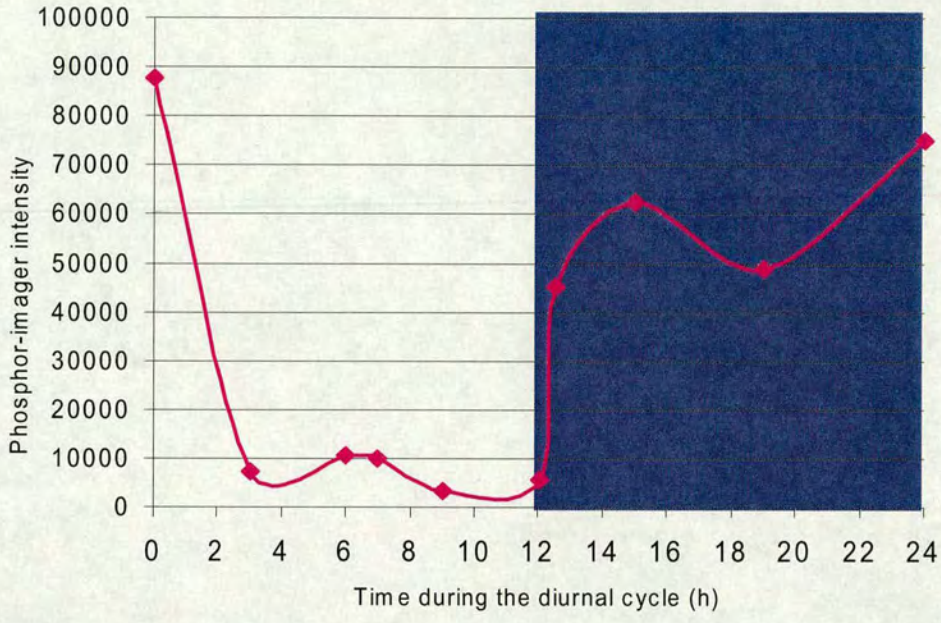
Col0 plantlets were grown in a L12 h:D12 h regime for 3 weeks. Plantlets were subsequently transferred to DD for 48 h and tissue samples were harvested every 3 h. Upon harvest the shoot and root tissues were separated (mRNA of the shoot tissue is displayed in figure 4.4.5). The *STP1* transcripts are displayed upon quantitation of the RNA gel-blot. Samples harvested during the subjective day are represented by a white background and during the subjective night by a blue background.

4.4.4 *STP1* gene expression in the arrhythmia *elf3* mutant

The expression of the *STP1* gene in the shoots of *elf3* mutant plantlets entrained to a L12 h:D12 h regime for 4 days (figure 4.4.7) displays a diurnal expression pattern similar to that seen in Col0 and Ws wild-type plantlets (Figure 4.2.1A and fig 4.2.4 respectively). The *STP1* transcript levels are displayed following quantification by phosphor-imaging (figure 4.4.7) of the RNA gel-blot (figure 4.4.7B). The *STP1* transcripts are low in the shoots of plantlets during the light phase, with a small increase after 6 h light. Upon entering the dark *STP1* transcripts accumulate and display a bimodal expression pattern in the shoots of plantlets harvested through the dark phase. Due to a lack of time-points it is not possible to accurately describe where the peaks in the *STP1* transcript level occur.

To investigate whether *STP1* gene expression displays an arrhythmic circadian rhythm in the *elf3* mutant, plantlets entrained to a L12 h:D12 h regime were subsequently transferred to LL and the *STP1* transcript level was monitored in shoot tissue (figure 4.4.8). At the end of the light phase *elf3* mutant plantlets were transferred to LL for 48 h; therefore 0-12 h and 24-36 h time-points represent subjective night and 12-24 h and 36-48 h subjective day. The *STP1* transcript level in the shoots was quantified by phosphor-imaging (figure 4.4.8A) of the RNA gel-blot (figure 4.4.8B). The *STP1* transcript level peaks at the beginning of the subjective day, after 12 h and 36 h LL, with a periodicity of 24 h. Therefore, *STP1* gene expression does appear to display a circadian rhythm in the *elf3* mutant, whereas other clock-controlled genes reported display arrhythmic expression (McWatter *et al.,* 2000). However, the peak in *STP1* transcripts in the subjective day is approximately 9 h earlier than wild-type (figure 4.4.1A) so the regulation of the *STP1*

A: STP1 transcripts following quantitation by phosphor-imaging



B: The RNA gel-blot of *STP1* transcripts in the shoots

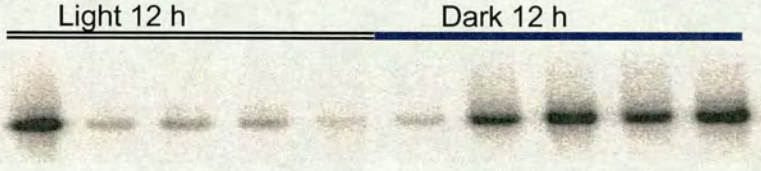
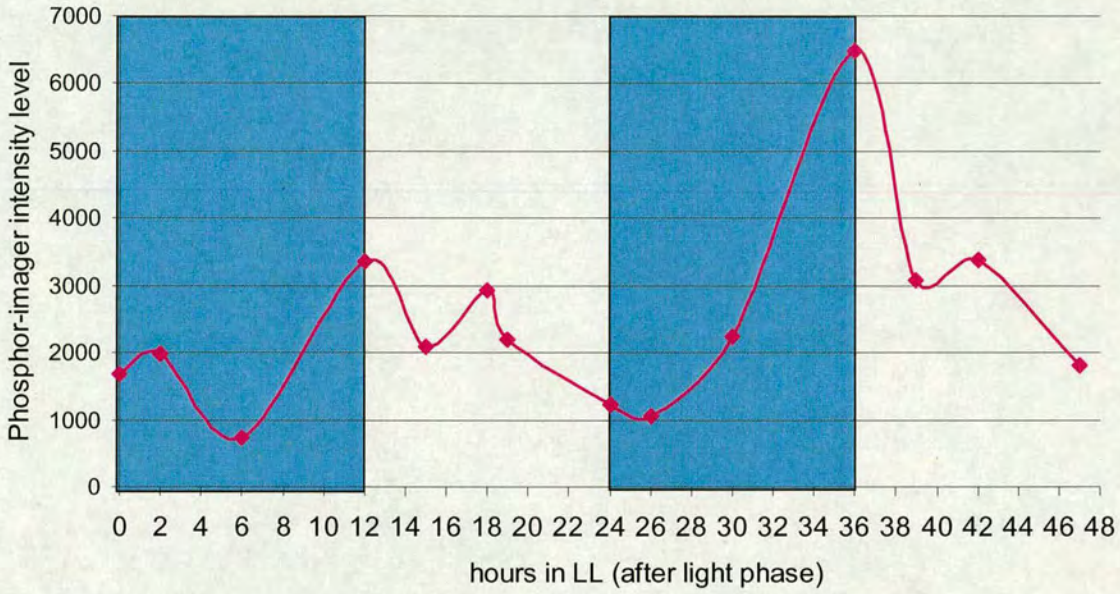


Figure 4.4.7 The expression of the *STP1* gene in the shoots of *elf3* mutant plantlets following a 4 day entrainment to a L12 h:D12 h regime

The *elf3* mutant plantlets were grown in continuous light for 8 days. Plantlets were then entrained to a L12 h:D12 h regime for 4 days. The *STP1* transcripts were quantified by phosphor-imaging (A) of the RNA gel-blot (B). Shoot samples harvested during the light phase are represented by a white background/bar and during the dark phase by a blue background/bar.

A: *STP1* transcripts following quantitation by phosphor-imaging



B: The RNA gel-blot of *STP1* transcripts in the shoots

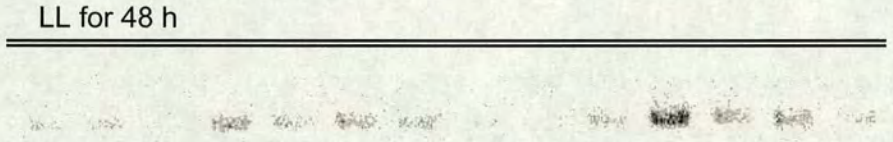


Figure 4.4.8 The expression of the *STP1* gene in the shoots of *elf3* mutant plantlets transferred to continuous light for 48 h following a 4 day entrainment to a L12 h:D12 h regime

Light-grown *elf3* mutant plantlets were entrained to a L12 h:D12 h regime. Plantlets were subsequently transferred to LL for 48 h and shoot samples were harvested every 3 h. The *STP1* transcripts were quantified by phosphor-imaging (A) of the RNA gel-blot (B). Samples harvested during the subjective day are represented by a white background and during the subjective night by a blue background.

is affected in the *elf3* plantlets, in a way that is not currently understood.

4.5 Sugar response of *STP1* gene expression in plantlets entrained to a L12 h:D12 h regime

4.5.1 Aims

When plants are entrained to a L12 h:D12 h regime *STP1* gene expression is low through the light phase, with a small increase in transcripts after 6 h light and upon subsequent transfer of plantlets to the dark the transcript level increases (refer to 4.2 for more detail). Furthermore, the *STP1* transcript level is repressed in plantlets treated with exogenous glucose in plantlets grown in continuous light (chapter 3). Therefore, it is possible the distinctive regulation of *STP1* gene through the diurnal cycle may be due, at least in part, to the carbohydrate status of the plantlets. The effect of exogenous glucose upon *STP1* gene expression in plantlets entrained to a diurnal cycle is investigated below. Plantlets were treated with either 0 mM, 3 mM or 30 mM glucose and shoot tissue was harvested from treated and untreated plantlets during the light and dark phases.

The impact of altered sugar content and metabolism upon *STP1* gene expression was also investigated, in the *sex4*, *pgm1* and *det3* mutants. The *sex4* mutant is defective in a chloroplastic endoamylase isoform. Therefore the mutant has a reduced capacity for starch degradation. During the night leaves have a high level of starch and during the day the partitioning of photosynthate to starch is reduced relative to wild-type (Zeeman and Ap Rees, 1999). The *pgm1* mutant is defective in the chloroplast isozyme phosphoglucomutase. During the day the leaves of the mutant accumulate high levels of soluble sugars and minimal levels of starch compared to wild-type (Caspar *et al.,* 1985). The *det3* mutant is defective in

vacuolar H^+-ATPase activity; consequently, sucrose is not loaded in to the vacuole and accumulates in the cytoplasm. Sucrose-inducible gene expression is high in mutant plants in the middle of the night during starch mobilisation (per. comm. Dr M. Campbell, University of Oxford, UK).

In addition, the effect of exogenous glucose upon the circadian regulation of *STP1* gene is also analysed.

4.5.2 The effect of exogenous glucose upon the diurnal expression of the *STP1* gene

Light phase

The effect of exogenous glucose upon the 6 h light phase peak of *STP1* transcripts during the diurnal cycle was investigated (figure 4.5.1). After 5 h in the light phase plantlets were treated with 0 mM and 30 mM glucose for 2 h. The *STP1* transcript level in the shoots of untreated and treated plantlets was quantified by phosphor-imaging of the RNA gel-blot. In untreated plantlets no apparent increase in *STP1* transcripts occurs mid-light phase, as seen previous in plantlets entrained to a L12 h:D12 h regime (figure 4.2.1A and figure 4.2.4). However, in plantlets incubated with water (0 mM glucose) at 6 h light a 2-fold increase in *STP1* transcripts occurs relative to that in untreated plantlets. It is possible that this increase in the transcript

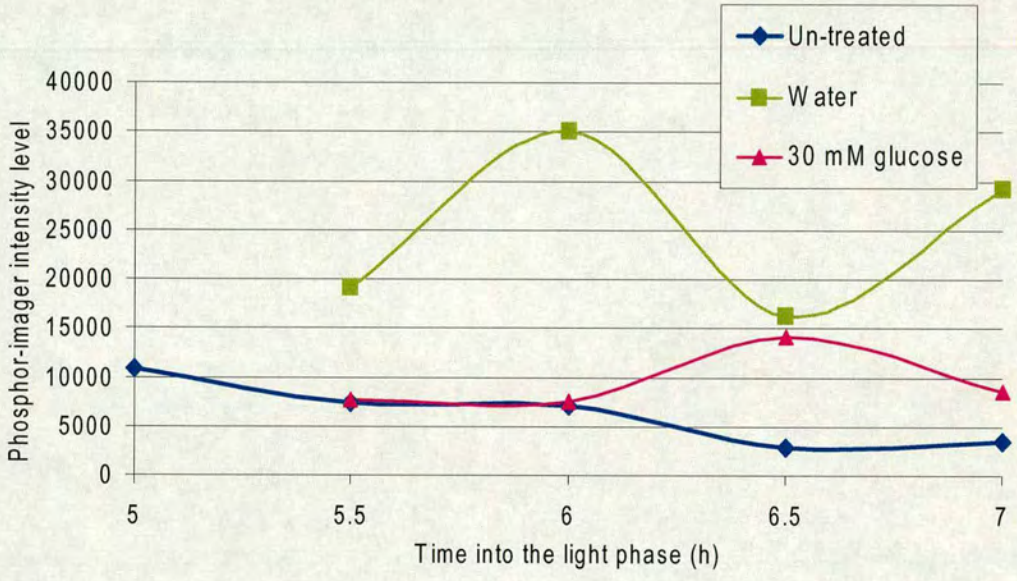


Figure 4.5.1 The glucose response of the *STP1* gene in plantlets entrained to a L12 h:D12 h regime

Col0 plantlets grown in continuous light for 10 days were entrained to a L12 h:D12 h regime for 4 days. Plantlets were treated with either water (green squares) or 30 mM glucose (pink triangles) 5 h after entering the light phase. The *STP1* transcript was subsequently monitored in the shoots of treated and untreated plantlets every 30 min for 2 h. *STP1* transcripts are displayed following quantification by phosphor-imaging of the RNA-gel-blot.

level is due to the induction of the *STP1* gene by mechano-stimulation, as previously described in light-grown plantlets (refer to section 3.3.6 for more detail). Treatment of plantlets with 30 mM glucose represses the accumulation of *STP1* transcripts relative to that observed in water-treated plantlets, whereas the transcript level is similar to that in untreated plantlets. This raises the possibility that in plantlets entrained to a L12 h:D12 h regime the *STP1* gene expression, during at least part of the light phase, is less responsive to exogenous glucose than that in light-grown plantlets (section 3.3). However, due to time restraints this experiment has not been repeated, so it cannot be said with certainty that this result is representative of the glucose response of the *STP1* gene in plantlets during the light. Further work is required to understand the regulation of the *STP1* gene by glucose through the light phase in plantlets entrained to a diurnal cycle.

Dark phase

The effect of exogenous glucose and light upon the dark accumulation of *STP1* transcripts in plantlets entrained to a L12 h:D12 h regime was investigated. Plantlets that were at the point of entering the dark phase (as plates were being wrapped in foil) were either maintained in the light, transferred the dark, or treated with 30 mM glucose prior to entering the dark. Shoot tissue was harvested prior to the treatment of the plantlets (0 h sample), and every 30 min during the first 4 h of the dark phase (treatment period). The *STP1* transcript level in the shoots of the treated and untreated plantlets is displayed following quantification of the RNA gel-blot by phosphor-imaging (figure 4.5.2). In untreated plantlets the *STP1* transcript level increases upon the transfer of plantlets to the dark, and peaks 1 h after the

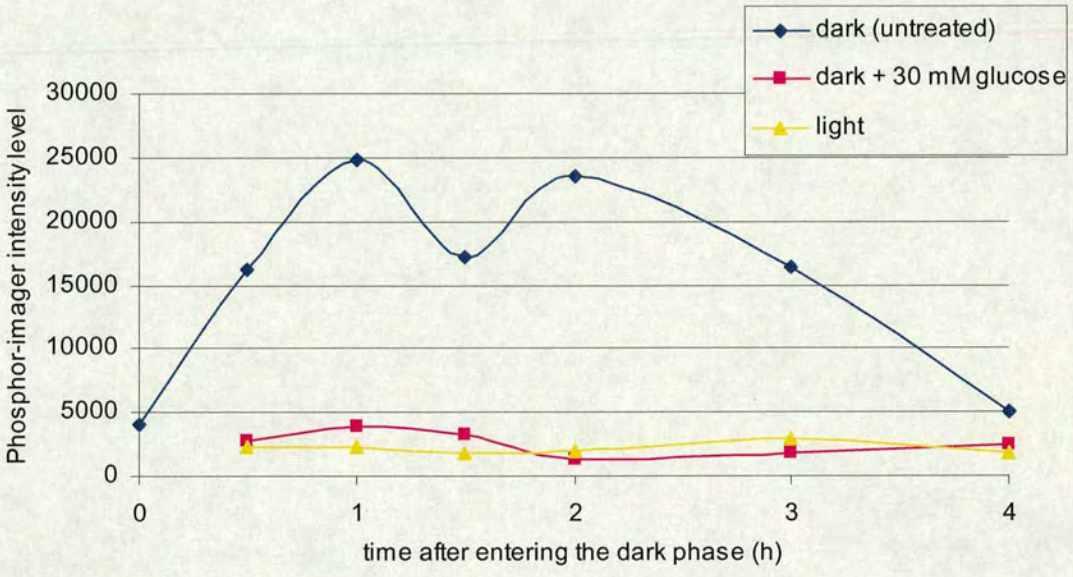


Figure 4.5.2 The response of *STP1* gene expression to glucose and light during the first 4 h of the dark phase in plantlets entrained to a L12 h:D12 h regime

Col0 plantlets grown in continuous light for 10 days were entrained to a L12 h :D12 h regime for 4 days. Upon transition from the light to the dark phase plantlets were treated with either 30 mM glucose (pink squares) or light (yellow triangles). The *STP1* transcript amounts were monitored in the shoots of treated and untreated (blue) plantlets for 4 h. *STP1* transcripts are displayed following quantification by phosphor-imaging of the RNA-gel-blot.

transition; the level remains relatively high up to 3 h into the dark, at which point the level decreases noticeably. The initial dark peak in *STP1* transcripts is typically seen between 1 h 30 min and 2 h after plantlets enter the dark (figure 4.2.1A and figure 4.2.4). No dark-accumulation of *STP1* transcripts occurs in the shoots of plantlets treated with 30 mM glucose, which suggests that the dark-induction of *STP1* transcripts is at least in part, a response to carbohydrate limitation. Light also represses the dark accumulation of *STP1* transcripts, but it is not clear if this is a response to light, the photosynthetic production of sugars or a combination of both. However, this result is consistent with the *STP1* gene expression in shoot tissue of plantlets transferred to LL following entrainment to a L12 h:D12 h regime, as the *STP1* transcript level remains low during the subjective night (figure 4.4.1).

A second experiment was conducted to address the effect of exogenous glucose upon the expression of the *STP1* gene during the complete 12 h dark phase (figure 4.5.3). To avoid submerging plantlets for an extended period of time (more than 5 h), the incubation of plantlets with either water or glucose was performed in 2 stages. Firstly, 0 mM and 30 mM glucose was added to plantlets during the light to dark transition (as plates were wrapped with foil) and shoot samples were harvested at intervals during the subsequent 4 h dark period (time points 0-4 h). Secondly, untreated plantlets incubated in the dark for 4 h were incubated with either 0 mM or 30 mM glucose, and shoot samples were harvested at intervals during the remainder of the dark phase (time points 5-12 h).

The *STP1* transcript level in the shoot tissue of untreated plantlets is similar to that seen in the shoots of plantlets monitored through the L12 h:D12 h cycle (Figure 4.2.1A). The *STP1* transcript level peaks 1 h 30 min after entering the dark; the level

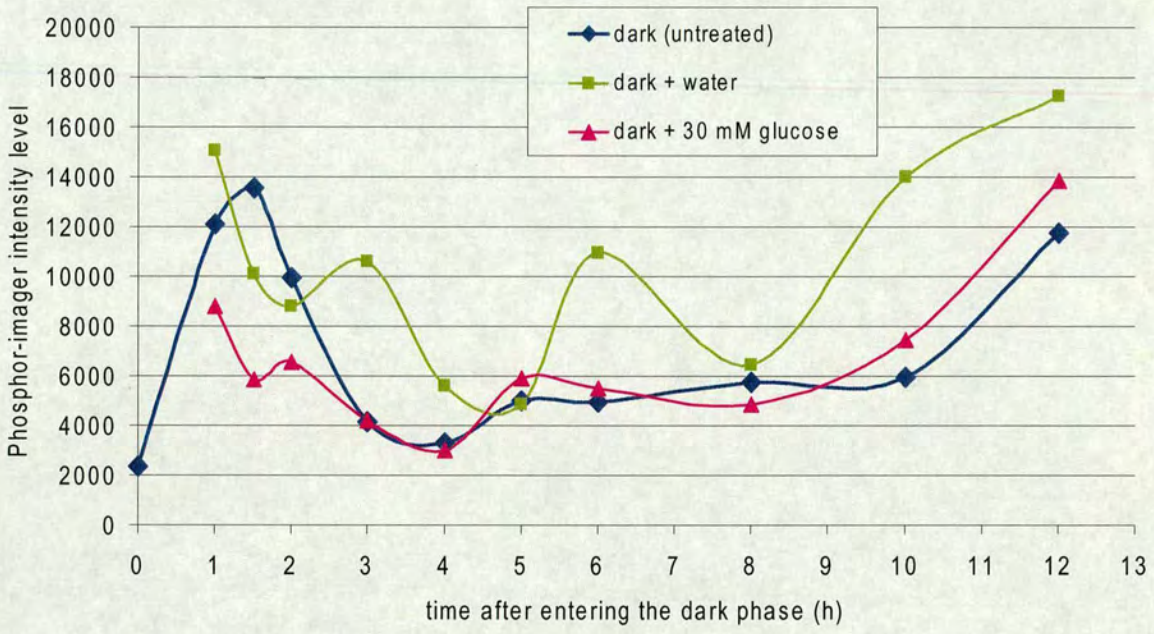


Figure 4.5.3 The glucose response of the *STP1* gene during the dark phase in plantlets entrained to a L12 h:D12 h regime

Col0 plantlets grown in continuous light for 10 days were entrained to a L12 h :D12 h regime for 4 days. Plantlets were treated with either water (green) or 30 mM glucose (pink) upon the transfer of plantlets from the light to the dark phase. The *STP1* transcript amounts were monitored in the shoots of treated and untreated (blue) plantlets throughout the dark phase of the diurnal cycle. *STP1* transcripts are displayed following quantification by phosphor-imaging of the RNA-gel-blot.

subsequently falls and remains low for approximately 8 h, and then increases towards the end of the dark phase. The level of the *STP1* transcripts in plantlets treated with water (0 mM glucose) is higher throughout the dark phase relative to that seen in untreated plantlets, with the exception of the first 2 h. This could be due to the effect of mechano-stimulation upon the expression of the *STP1* gene. The initial dark-accumulation of *STP1* transcripts is repressed in plantlets treated with 30 mM glucose relative to that seen in water-treated plantlets, whereas the level is similar to that in untreated plantlets. This is the only stage during the dark phase where *STP1* gene expression is appreciably repressed by glucose. This is consistent with the repression of the initial dark-accumulation of *STP1* transcripts by glucose, described above (figure 4.5.2). This raises the possibility that the expression of the *STP1* gene after the first 2 h of the dark phase is less responsive to exogenous glucose. Due to time constraints this experiment has not been repeated, so at present it cannot be said with certainty that the second dark-accumulation of *STP1* transcripts is insensitive to glucose.

4.5.3 *STP1* gene expression in sugar metabolism mutants

The expression of the *STP1* gene was investigated in mutants with altered sugar content or metabolism relative to wild-type. The diurnal expression pattern of the *STP1* gene in the *sex4*, *pgm1* and *det3* mutants is compared to that of Col0, following the entrainment of plantlets to a L12 h:D12 h regime for 4 days (figure 4.5.4). The 0 h shoot sample was harvested approximately 10 min before the plantlets were transferred to the light at the beginning of the experiment, therefore the 0 h time-point represents tissue from plantlets exposed to 12 h dark only, prior to harvest. The 24 h shoot sample was harvested after 12 h dark at the end of the

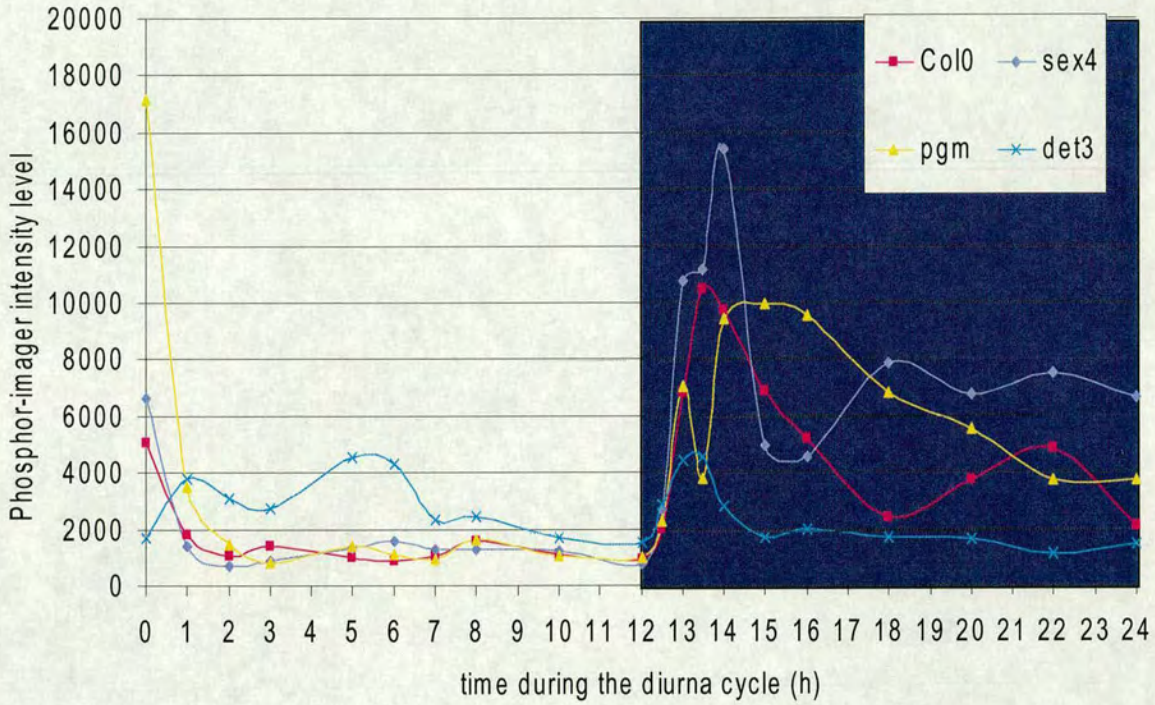


Figure 4.5.4 The expression of the *STP1* gene during the diurnal cycle in the shoots of wild-type and mutants with altered sugar content

Col0 (pink squares), *sex4* (blue diamonds), *pgm1* (yellow triangles) and *det3* (turquoise crosses) plantlets grown in continuous light for 10 days were entrained to a L12 h:D12 h regime for 4 days. *STP1* transcripts are displayed following quantification by phosphor-imaging of the RNA-gel-blot. Shoot samples harvested during the light phase are represented by a white background and during the dark phase by a blue background.

experiment; these samples may have been exposed to low levels of light prior to collection. The 12 h shoot sample was harvested 5 min before plantlets were transferred to the dark.

The diurnal expression pattern of the *STP1* gene in the shoot tissue of Col0, *sex4* and *pgm1* is similar, in that the *STP1* transcript levels are low throughout the light and high in the dark. However, the timing and magnitude of the *STP1* transcript levels in the various plantlets is different during the dark phase. The initial peak in the shoot-specific *STP1* transcript level following the transfer of plantlets to the dark occurs after 1 h in *pgm1*, 1 h 30 min in Col0 and 2 h in *sex4*. The magnitude of the dark-peak in *STP1* transcripts, at these time points, is higher in *pgm1* mutants than that in Col0; and lower in *sex4* mutants compared to that in Col0.

In the shoots of the *sex4* and Col0 plantlets, the *STP1* transcript level then falls until the end of the dark phase, when a second accumulation in the transcript level is seen. In *sex4* plantlets the *STP1* transcript level is higher relative to that seen in wild-type (Col0) throughout the dark. The *STP1* transcript level in the *pgm1* mutant during the remainder of the dark is different to that in the shoots of Col0 and *sex4* plantlets. Following the initial peak in the *STP1* transcript level after 1 h dark, *STP1* transcription increases again and plateaux 2 h to 4 h after entering the dark phase. Subsequently, the *STP1* transcript level decreases for the remainder of the phase. Although the *STP1* transcript level in the shoots of *pgm1* mutant plantlets prior to entering the light phase (time point 0 h) is the highest level observed throughout this experiment.

The diurnal pattern of the shoot-specific *STP1* gene expression in the *det3* mutant is distinct from that observed in wild-type, *sex4* and *pgm1* plantlets. The *STP1* transcript level observed during the first 7 h of the light phase and that seen during

the first 2 h of the dark phase are similar in magnitude, whereas in the shoots of wild-type plantlets the difference in the *STP1* transcript level between those incubated in the light or transferred to the dark is approximately 10-fold. In the shoot tissue of *det3* plantlets, the *STP1* transcripts are higher in the light and lower in the dark than that observed in the other ecotypes. For example, in *det3* mutants the peak in *STP1* transcripts after 5 h light is 3 times higher than that observed in *sex4*, *pgm1* and Col0. For the remainder of the light phase, the *STP1* transcript level in the *det3* mutant is low. Upon entering the dark, a small induction of *STP1* transcription is seen for 2 h. During the remainder of the dark phase the *STP1* transcript level is appreciably lower than that of the other ecotypes.

The differences in the diurnal expression of the *STP1* gene in the shoot tissue of the various plantlets may be due to differences in sugar content relative to wild-type. However, due to time-constraints the sugar content of the mutants described above has not been measured. Completion of this in the future may link changes in sugar content to the regulation of *STP1* transcripts in the mutant and wild-type plantlets.

Furthermore, the expression of the *STP1* gene in the *det3* mutant is distinct from that seen in *Col0*, *sex4* and *pgm1*. It is not clear why *STP1* gene expression is appreciably different in the *det3* mutant. It may be a result of altered sugar content, or possibility linked to defective vacuolar H^+-ATPase activity. More experimentation is required to identify any factors that may be responsible for the differential regulation of *STP1* gene expression in the *det3* mutant relative to wild-type.

4.5.4 The effect of exogenous glucose upon the circadian regulation of the *STP1* gene

The expression of the *STP1* gene in plantlets grown in continuous light is repressed by exogenous glucose (section 3.3). The glucose response of the *STP1* gene in

plantlets entrained to a diurnal cycle is less clear (section 4.5.2). To investigate the effect of exogenous glucose upon the circadian rhythm of the *STP1* transcript level, plantlets entrained to a L12 h:D12 h regime were subsequently treated with or without 10 mM glucose and transferred to LL (figure 4.5.5) or DD (figure 4.5.6). The shoot tissue of treated and untreated plantlets was subsequently harvested every 3 h for 48 h, RNA was prepared and Northerns performed. The *STP1* transcript level was quantified by phosphor-imaging of the RNA gel-blot.

Plantlets were transferred to LL for 48 h at the end of the light phase. Therefore 0-12 h and 24-36 h time-points represent subjective night and 12-24 h and 36-48 h subjective day. The circadian expression of the *STP1* gene is seen in both untreated and glucose-treated plantlets during the subjective day (figure 4.5.5). In untreated plantlets transferred to LL, the *STP1* transcript peaks occur after 21 h and 42 h light. This result is consistent with the view that *STP1* gene expression in the shoot tissue displays a circadian rhythm in LL, which presumably corresponds to the 6 h light phase *STP1* transcript peak of the diurnal cycle (figure 4.2.1A).

In plantlets incubated with 10 mM glucose the *STP1* transcript level during the subjective day is more than 2-fold higher than that observed in untreated plantlets. It is possible that the increase in *STP1* transcripts in treated plantlets is an effect of mechanical stimulation. Furthermore, the increase in *STP1* transcripts is restricted to the subjective day (12-24 h and 36-48 h) and is not apparent in the subjective

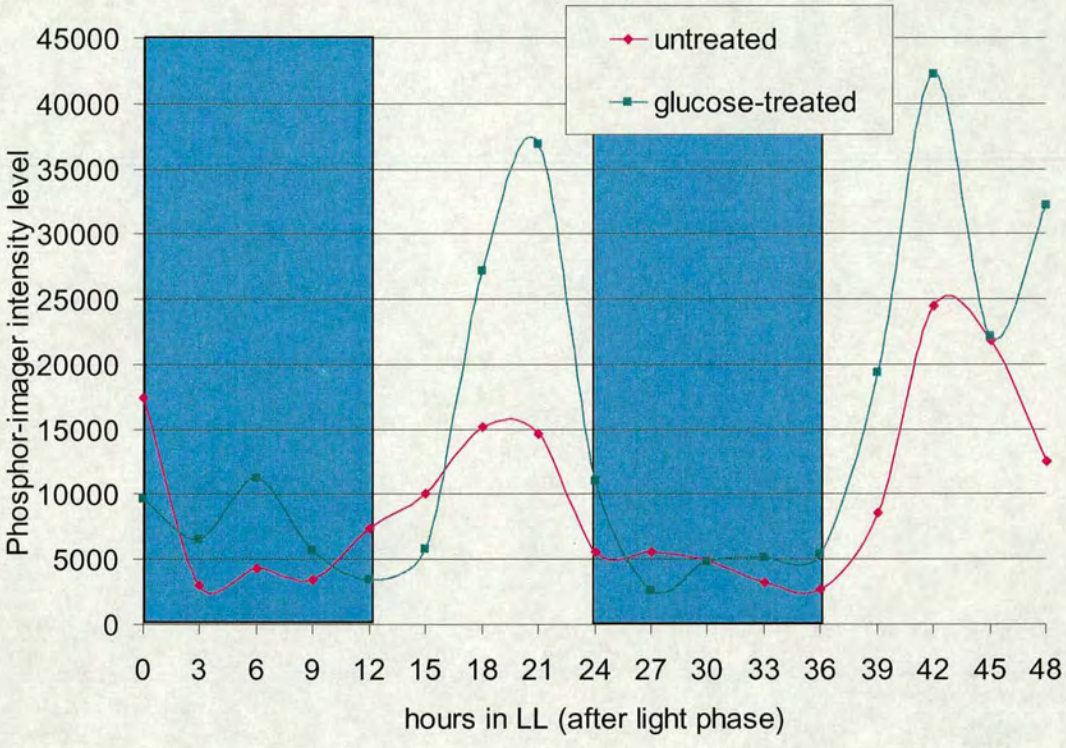


Figure 4.5.5 The glucose response of the circadian expression of the *STP1* gene in continuous light in the shoots of plantlets previously entrained to a L12 h:D12 h regime

Col0 plantlets grown in continuous light for 10 days were entrained to a L12 h :D12 h regime prior to continuous light treatment. The *STP1* transcripts were monitored in the shoots of plantlets incubated with (green) and without (pink) 10 mM glucose every 3 h in LL. Samples harvested during the subjective day are represented by a white background and during the subjective night by a blue background.

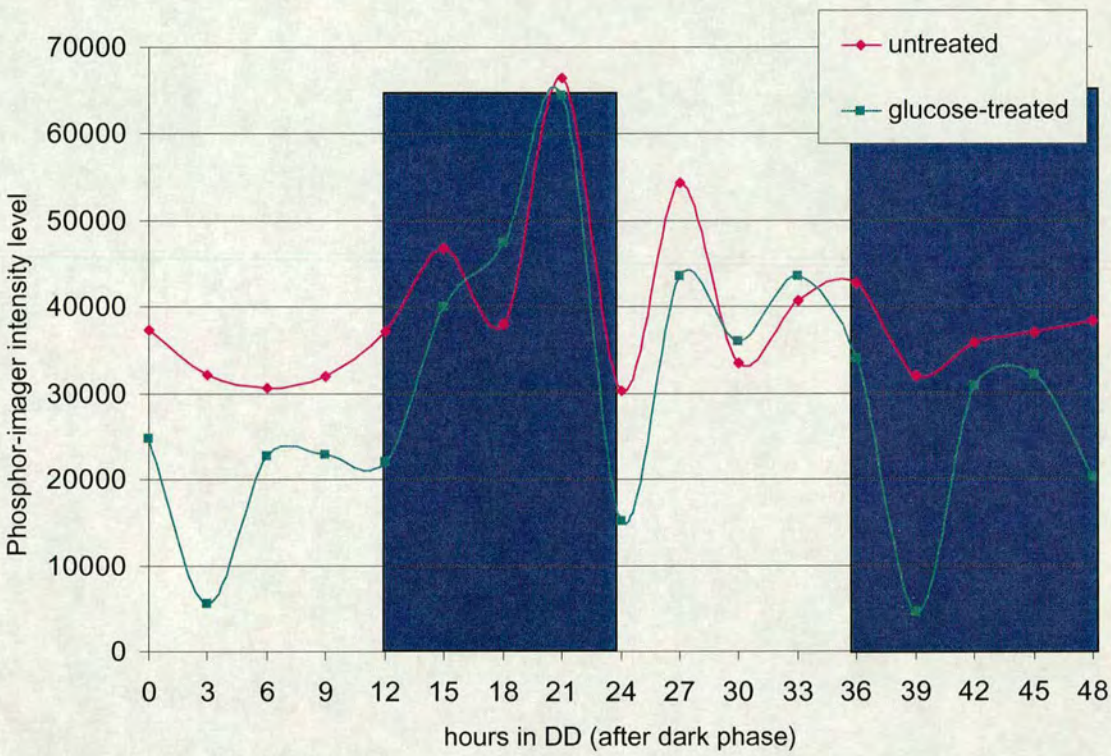


Figure 4.5.6 The glucose response of the circadian expression of the *STP1* gene in continuous dark in the shoots of plantlets previously entrained to a L12 h:D12 h regime

Col0 plantlets grown in continuous light for 10 days were entrained to a L12 h: D12 h regime. The *STP1* transcripts were monitored in the shoots of plantlets incubated with (green) and without (pink) 10 mM glucose every 3 h in DD. Samples harvested during the subjective day are represented by a white background and during the subjective night by a blue background.

night (0-12 h and 24-36 h). This result is consistent with mechano-stimulation of the *STP1* gene, which appears to be particularly sensitive in the light (see 3.3.6). However, as the experiment lacks a water control it cannot be said with certainty whether the increase in *STP1* transcripts in treated plantlets is due to the inadvertent mechanical stimulation of plantlets caused by the addition of the glucose solution, or a direct or indirect effect of glucose upon the metabolic pathways and gene regulation of the plantlet.

The effect of exogenous glucose upon the circadian rhythm of *STP1* gene was also investigated in the shoots of plantlets transferred to DD (figure 4.5.6). Plantlets were entrained in a L12 h:D12 h regime for 4 days and were transferred to DD for 48 h at the end of the dark phase; therefore 0-12 h and 24-36 h time-points represent subjective days and 12-24 h and 36-48 h subjective nights. The shoot-specific expression of the *STP1* gene in plantlets treated with 10 mM glucose and DD reveals that the sensitivity of *STP1* transcripts to glucose may vary depending on the time of 'day'. For example, the greatest repression of the *STP1* transcript level by 10 mM exogenous glucose is in the shoots of plantlets harvested during the first subjective day (0 h to 12 h DD), upon entering the next consecutive subjective days (24 h and 48 h DD), and at the beginning of the subjective nights (12 h and 39 h DD). The *STP1* transcript level appears to be less sensitive to glucose in the shoots of plantlets harvested during the subjective night. A shortcoming of this experiment is the lack of 0 mM (water) controls, particularly in light of the discovery that water/sugar treatments can result in the mechano-stimulation of *STP1* gene expression. Therefore, it is difficult to interpret this data. However, the *STP1* transcript level in the glucose-treated plantlets is not appreciably higher than that in untreated plantlets, unlike *STP1* transcript level in glucose-treated plantlets in LL (figure 4.5.5). This experiment needs to be repeated, including samples from water-

treated plantlets, to identify the response of the circadian expression of the *STP1* gene to mechanical stimulation and/or exogenous glucose.

4.6 Conclusion

The diurnal expression of the *STP1* gene

The expression of the *STP1* gene following the entrainment of plantlets to a diurnal regime (section 4.2) is more complex than that observed in light-grown plantlets (section 3.2). However, in the shoots of plantlets grown both in continuous light and transferred to the dark, or in a diurnal regime, the *STP1* transcript level accumulates appreciably upon exposure to the dark. The complexity of *STP1* gene expression in plantlets entrained to a diurnal cycle is reflected by the differences in the magnitude and timing of expression through the light and dark phases. It appears likely that the diurnal regulation of the *STP1* gene is affected by day-length and sugar content.

When plantlets are entrained to L12 h:D12 h regime the *STP1* gene in the shoots and roots displays distinct patterns of expression (figure 4.2.1A and 4.2.3 respectively). This may be because the *STP1* gene responds differently to biotic and abiotic factors in each tissue. For example, the *STP1* transcript level appears to be more responsive to light in the shoots than in the roots. It is possible that such differences in *STP1* transcripts may be indicative of different roles of the STP1 transporter in each tissue. This is consistent with a different pattern of STP 3-OMG transport activity in the shoots and roots of plantlets entrained to a diurnal cycle (figure 4.3.2); the majority of which can be attributed to STP1 activity (figure 4.3.5).

The *STP1* transcript level in both the shoots and roots peaks 1 h to 2 h after entering the dark phase in wild-type plantlets. However, the relative dark-

accumulation of *STP1* transcripts is over 5 times greater in shoots than that in roots (figure 4.2.1A and 4.2.3). In fact, the *STP1* transcript level observed in the roots 1 h after entering the dark is comparable to that observed in the shoots during the light. The shoot-specific peak in *STP1* transcripts within 2 h of entering the dark is seen in all the growth regimes, mutants and ecotypes monitored in this report (section 4.2). Therefore, it appears that this facet of *STP1* gene expression is important in the diurnal regulation of the principal *STP* gene in *Arabidopsis*.

The accumulation of *STP1* transcripts within the first 2 h of the dark phase is also seen in mutants with reportedly altered sugar content relative to wild-type (figure 4.5.4). However, the magnitude of the transcript level does vary. For example, the *STP1* transcript level is higher in the starch-excess (*sex4*) mutant than in the starch deficient/high glucose (*pgm1*) mutant. Therefore, the sugar content of such plantlets may alter the responsiveness of *STP1* gene expression at specific times through the diurnal cycle. This is consistent with the repression of the initial dark accumulation of *STP1* transcripts in wild-type plantlets treated with 30 mM exogenous glucose (figure 4.5.2 and 4.5.3).

The timing of the initial dark peak is similar whether plantlets are entrained to L12 h:D12 h, L4 h:D20 h or L20 h:D4 h regimes, which suggests that possibly the exposure of plantlets to darkness is required to trigger the *STP1* transcript accumulation, independently of day-length (figures 4.2.1A, 4.2.5 and 4.2.6). If this is the case, it is probable that light signalling plays a role in the dark accumulation of *STP1* transcripts. Furthermore, this is consistent with the absence of any transcript accumulation in entrained plantlets that were not transferred to the dark phase, but were maintained in the light at the end of the light phase. This implies that entrainment of plantlets to a diurnal cycle is not sufficient for the dark-expression pattern of the *STP1* gene to occur in the subjective night (figure 4.4.1A).

The analysis of Col0 plantlets entrained to different light regimes (L12 h:D12 h, L20 h:D4 h and L4 h:D20 h) also reveals that *STP1* gene expression does not 'anticipate' the light to dark or the dark to light transitions (figures 4.2.1A, 4.2.5 and 4.2.6); as the *STP1* transcript levels do not accumulate prior to the transfer of plantlets to the dark, or fall before transfer to the light. Furthermore, by comparing the timing of peaks in the *STP1* transcript level in plantlets entrained to different growth regimes, the role of day-length can be examined. For example, the timing of the second accumulation in *STP1* transcripts observed in plantlets towards the end of the dark phase, appears to be dependent on the diurnal growth regime plantlets were entrained with. The second accumulation of *STP1* transcripts in the dark phase occurs after 3 h dark in plantlets entrained to a L20 h:D4 h regime, after 6 h in a L12 h:D12 h regime and after 10 h in a L4 h:D20 h regime. Therefore, it is possible that the timing of *STP1* gene expression is at least in part, regulated by day-length. It is unlikely that such differences are due to experimental variation, as all the plantlets used in this experiment were grown, maintained and treated in the same way and the shoot samples were harvested at the same time.

Moreover, the glucose response of the *STP1* transcript level within the first 2 h of entering the dark phase is different from that observed towards the end of the dark phase. In preliminary experiments the *STP1* transcript level in plantlets treated with 30 mM glucose is repressed compared to that in untreated plantlets during the first 2 h of the dark phase (figure 4.5.2 and figure 4.5.3); whereas, during the remainder of the dark phase the *STP1* transcript levels in untreated and glucose-treated plantlets are similar. In addition, the *STP1* transcripts in plantlets treated with water during the latter stages of dark phase are higher than that in untreated plantlets (figure 4.5.3). It is possibly that this increase in *STP1* gene expression is a result of inadvertent mechanical stimulation of treated-plantlets, which has previously been shown to

regulate *STP1* gene expression (detailed in section 3.3.6). Although, the effect of flooding, lack of oxygen and stress upon *STP1* gene expression cannot be ruled out.

The level of *STP1* transcripts during the dark phase fluctuates, irrespectively of the light regime. This is most notable in the shoots of plantlets that were incubated in the dark for an extended time; for example, upon transfer of light-grown plantlets to 72 h dark (figure 3.2.1) or upon entrainment of plantlets to a L4 h:D20 h regime (figure 4.2.5). The rapidity of the fluctuations in the *STP1* transcript level suggests that the accumulation and turn-over of *STP1* transcripts may be tightly controlled by mRNA stability, as well as, transcriptional regulation.

To conclude, the dark phase expression pattern of the *STP1* gene appears to be regulated in a multi-faceted manner. For example, the initial peak of *STP1* transcripts, upon entering the dark is probably due to the combination of the changing carbohydrate status within the plantlet and direct exposure to darkness. Whereas, the second dark accumulation of the *STP1* transcript level appears to be less sensitive to glucose and the timing of the peak may be dependent on day-length or other physiological factors resulting from an altered photoperiod.

The expression of *STP1* gene in the shoot and root tissue of plantlets incubated in the light is low, irrespective of whether plantlets, are grown in continuous light or a diurnal cycle (figure 3.2.1 and figure 4.2.1A respectively). However, in the shoots of plantlets entrained to a L12 h:D12 h and L20 h:D4 h regime, a small peak in *STP1* transcripts occurs between 5 h and 9 h in the light phase (figure 4.2.1A and figure 4.2.6 respectively). Whereas, in plantlets entrained to a L4 h:D20 h regime, no accumulation of *STP1* transcripts is evident during the 4 h light phase (figure 4.2.5). Taken together these results suggest that the peak in the *STP1* transcript level during the light phase, occurs only after a specific number of hours in the light (more

than 5 h); and therefore the transcript level is likely to be regulated independently of day-length. A similar level of *STP1* gene expression is seen during the majority of the light phase in the roots of plantlets entrained to a L12 h:D12 h regime (figure 4.2.3).

In a preliminary experiment investigating the glucose response of *STP1* transcripts in plantlets during the light phase, plantlets were treated with water as a control, which in fact resulted in an increase in shoot-specific *STP1* transcripts relative to untreated and glucose-treated plantlets (figure 4.5.4). This is probably a result of mechano-stimulation, but the effect of stress, lack of oxygen or flooding cannot be ruled out. The effect of water upon *STP1* gene expression in the shoots of plantlets during the diurnal cycle (figure 4.5.3 and figure 4.5.4) is consistent with that seen in plantlets grown in continuous light, where either water or direct mechanical stimulation results in the induction of *STP1* gene expression (section 3.3.6).

To conclude, the light phase expression of *STP1* transcript levels appears to be regulated independently of day-length and glucose; and only after 5 h light exposure is an appreciable accumulation of *STP1* transcripts seen. Furthermore, the response of the *STP1* gene to mechano-stimulation is apparently greater during the light phase compared to that seen in the dark (figure 4.5.4. and fig 4.5.3 respectively; section 3.3.6).

Another approach employed to identify the sugar response of *STP1* gene expression, was the use of mutants impaired in sugar content and/or metabolism (figure 4.5.4). The comparison of the diurnal expression of the *STP1* gene in shoot tissue of Col0, *sex4*, *pgm1* and *det3* plantlets reveals differences in the timing and magnitude of the *STP1* transcript levels. It is unlikely such difference may be attributed to experimental variation, as all the plantlets were grown, maintained and

treated experimentally in the same way and the shoot samples were harvested at the same time. Therefore, the marked differences in *STP1* gene expression between the mutants and wild-type is likely to be a result of altered metabolic status and/or sugar content. However, the sugar content of these mutants has not been measured under the exact growth conditions used here, so no direct correlations can be made between the altered sugar content and the difference in *STP1* gene expression.

Furthermore, the expression of the *STP1* gene in the *det3* mutant is distinct from that seen in Col0, *sex4* and *pgm1*, in that 2 h after entering the dark phase *STP1* transcripts remain at a low level relative to that in wild-type plantlets and during the light phase the transcript level is appreciably higher than that in wild-type. It is not clear why *STP1* gene expression is appreciably different in the *det3* mutant. It may be a result of altered sugar content, or possibility linked to defective vacuolar H^+-ATPase activity. More experimentation is required to characterise any differential regulation of *det3* mutant relative to wild-type. However, as the *det3* mutant is defective in organ specific cell elongation and has a reduced response to brassinosteroids (Schumacher *et al.,* 1999), it is tempting to consider such future work may identify co-ordinated changes in the regulation of *STP1* gene expression and that of cell expansion. Hypothesis as to the possible functions of the STP1 monosaccharide transporter, including a role in the salvage of sugars during cell wall turnover and expansion is discussed in chapter 5.

Circadian rhythm of the *STP1* gene expression

In the shoot of plantlets entrained to a L12 h:D12 h regime and subsequently transferred to LL, a peak in *STP1* transcripts is seen 9 h into the subjective day that displays a periodicity of 24 h (figure 4.4.1). This suggests that *STP1* gene

expression in the shoots displays a circadian rhythm in LL that may correspond to the 5-7 h peak in *STP1* transcripts seen during the light phase in plantlets entrained to a diurnal cycle (figure 4.2.1A). The timing of the circadian rhythms of the *STP1* and *CAB* genes in LL are similar, even though in plantlets entrained to a diurnal cycle the peak in *CAB* transcripts occurs during the light, whereas *STP1* transcripts peak during the dark. The addition of 10 mM glucose to plantlets transferred to LL has no effect on the timing of the *STP1* circadian rhythm but increases the magnitude of *STP1* transcripts (figure 4.5.6). This is possibly due to inadvertent mechanical stimulation rather than the glucose treatment. Particularly as the *STP1* gene expression in glucose-treated plantlets relative to that in untreated plantlets also increases, during the L12 h:D12 h cycle (section 4.2). This is also consistent with the effect of low concentrations of glucose upon the expression of the *STP1* gene, which elicits an inductive response in plantlets grown in continuous light (figure 3.3.2).

An unexpected finding observed in plantlets entrained to a L12 h:D12 h regime upon subsequent transfer to LL, was that no accumulation of the *STP1* transcript level was apparent during the subjective night. This is unexpected because in plantlets entrained to a L12 h:D12 h regime, the greatest *STP1* transcript level occurs during the dark phase. Therefore, it is probable that entraining plantlets to a L12 h:D12 h regime is not sufficient for a circadian peak in *STP1* transcripts to occur in the subjective night in plantlets transferred to LL. This is consistent with the idea that exposure to darkness is required to initiate the dark accumulation of *STP1* transcripts in plantlets (discussed above). A theory that would explain the absence of any *STP1* transcript accumulation in the subjective night is that the *STP1* gene may become uncoupled from the 'biological clock' upon transfer to LL and thus only light-specific expression patterns of the *STP1* gene persist.

In addition, the magnitude of the light and glucose repression of the *STP1* transcript level during the dark phase is different in the shoots of plantlets entrained to a L12 h:D12 h regime and subsequently transferred to LL. For example, the *STP1* transcripts during the subjective night are repressed (figure 4.4.1), whereas in glucose-treated plantlets no obvious repression of *STP1* transcripts occurs through the majority of the dark phase (figure 4.5.3). These results highlight the complexity of *STP1* gene regulation, for it appears likely that the magnitude of transcript repression may vary depending on both the treatment and time of treatment. This is consistent with sugar and light modulating *STP1* gene expression via independent signalling pathways (section 3.4.3)

In plantlets grown for 3 weeks in a growth room set to a L12 h:D12 h regime and subsequently transferred to DD, *STP1* transcript levels display a circadian rhythm (figure 4.4.5). The shoot-specific accumulation of *STP1* transcripts occurs ~6 h in to the subjective night with a periodicity of approximately 24 h. Presumably this corresponds to the dark accumulation of *STP1* transcripts observed in the dark phase of plantlets grown in a L12 h:D12 h regime (figure 4.2.1A). The circadian expression pattern of the *STP1* gene in DD is restricted to the subjective night, as the *STP1* transcript level is low during the subjective day. This is consistent with the circadian regulation of the *STP1* gene in plantlets transferred to LL, where transcripts accumulated only in the subjective day (discussed above).

Interestingly, the circadian expression of the *STP1* gene upon transfer of plantlets to DD is not seen in plantlets entrained for 4 days to a L12 h:D12 h regime (figure 4.4.3 and figure 4.4.4). A possible reason for this is that 4 days is not sufficient to entrain *STP1* gene expression, although this is unlikely because 4 days entrainment is sufficient for the circadian expression of the *STP1* gene in plantlets transferred to LL to occur. Alternatively, wrapping plates in aluminium foil may result in a dark-induced accumulation of *STP1* transcripts that masks any rhythm. Whereas, the

circadian rhythm may be seen in plantlets maintained in a growth room because the *STP1* transcript level is lower, which could be due to the consistent exposure of plantlets to low levels of light during the dark phase, such as the leakage of light into the growth room under the door or through ventilation ducts.

Another unusual feature of the regulation of *STP1* gene expression is that the circadian peak in *STP1* transcripts is restricted to the subjective day under LL, and the subjective night under DD. It is possible that the *STP1* gene may become uncoupled from the 'biological clock' upon transfer to constant environmental conditions. Plantlets require environmental cues, such as the light to dark transition to continually reset the endogenous clock; without such cues the output pathways from the clock may become out of phase with the normal light-dark cycle. Therefore, upon transfer of plantlets to DD only the dark-specific expression patterns of the *STP1* gene persist.

The expression of the *STP1* gene in the shoots of *elf3* arrhythmia mutant plantlets entrained to a L12 h:D12 h regime and subsequently transferred to LL reveals a circadian rhythm (figure 4.4.8). The peak in *STP1* transcripts is observed at the beginning of the subjective light and has a period of 24 h. Therefore, *STP1* gene expression displays a circadian rhythm in the *elf3* mutant that is not arrhythmic, unlike other clock-controlled genes investigated in the *elf3* mutant, such as the *CAB* gene (McWatters *et al.,* 2000). However, the peak of *STP1* transcripts in the subjective day is 9 h earlier than that seen in wild-type (figure 4.4.1) so the regulation of *STP1* gene is affected in *elf3* plantlets, in a manner that is not currently understood. However, this raises the possibility that circadian rhythm of the *STP1* gene is less dependent on ELF3 than that of other clock-controlled genes. The ELF3 receptor is thought to function in light perception and in-put into the biological clock (Carre, 2002). Furthermore, the possibility of an *elf3*-resistant clock implies that

perhaps the oscillator controlling the circadian expression of the *STP1* gene is not light dependent. There is a postulated metabolic oscillator in *Kalanchoe* (Nimmo *et al.,* 2001; Bohn *et al.,* 2001); and examples of clock-controlled genes entrained by temperature fluctuations described by Kreps and Simon (1997).

An additional line of evidence that circadian regulation of *STP1* gene may not be dependent on a light-driven clock is the circadian rhythm of *STP1* transcripts in the roots of plantlets transferred in DD (figure 4.4.6). This suggests that perhaps the clock regulating *STP1* is not entrained by light perception, as photoreceptors are generally localised to green tissue. Intriguingly, the peaks in the *STP1* transcript level in the roots of plantlets grown in a L12 h:D12 h regime and subsequently transferred to DD occur in both the subjective day and night (figure 4.4.6). It is possible that in the absence of light perception and signalling in this organ, the *STP1* transcript levels observed in both the light and dark phases of a diurnal cycle correspond to the circadian rhythm upon transfer to continuous conditions. It is possible that *STP1* gene expression displays such circadian rhythm, as no light signal from environment cues can be perceived to subsequently modulate *STP1* transcript levels in the roots.

Glucose transport activity during the diurnal cycle

The level of 3-OMG uptake by plantlets entrained to a diurnal cycle is higher than that seen in plantlets grown in continuous light (figure 4.3.1 and figure 3.5.1A respectively). Furthermore, the pattern of 3-OMG uptake changes between the shoots and roots throughout the diurnal cycle (figure 4.3.4), whereas the uptake by light-grown plantlets under both light and dark treatment is predominately localised to the roots (figure 3.5.2 and figure 3.5.3).

The uptake of 3-OMG by Ws through the L12 h:D12 h cycle was investigated so that direct comparisons could be made between wild-type and the *stp1* mutant (Ws ecotype) (figure 4.3.5). The pattern of 3-OMG uptake through a diurnal cycle by Ws plantlets is similar to that by Col0 (figure 4.3.5 and 4.3.1 respectively). Furthermore, the *STP1* gene expression in the shoots of Ws plantlets during the diurnal cycle resembles that seen in Col0 (figure 4.2.4 and figure 4.2.1A respectively). A high level of 3-OMG uptake is seen by Ws plantlets through the light phase, which falls towards the end of the phase. The level of uptake by plantlets in the dark phase is also high, the maximum rate of 3-OMG uptake results 6 h after entering the dark. The uptake of 3-OMG by *stp1* mutant plantlets remains constant throughout the diurnal cycle; the rate of uptake is up to 70% lower than that by wild-type plantlets, with the exception of the 9 h light time-point when the uptake of 3-OMG is similar in both Ws and *stp1* mutant. 3-OMG uptake by wild-type plantlets represents the collective transport activity of all the STP transporters. Therefore, it is likely that the transport activity of one or more of the remaining STPs accounts for the 3-OMG uptake at the end of the light phase, rather than STP1. For example, *STP4* transcripts have previously been localised to the root tip, and Col0 has high 3-OMG uptake activity by the roots at the end of the light phase. The results described above are consistent with previous reports that STP1 is the major plasma membrane glucose transporter in *Arabidopsis* plantlets (Sherson *et al.,* 2000). Consequently, the fluctuations in 3-OMG uptake observed in plantlets throughout the diurnal cycle are principally the result of STP1 transport activity.

The pattern of 3-OMG transport activity attributed to STP1 (section 4.3) does not resemble the expression pattern of the *STP1* gene (section 4.2) in plantlets entrained to a L12 h:D12 h regime. An explanation to account for such differences is that the synthesis and activity of the STP1 protein could be due to modification downstream of mRNA, such as translational or post-translational processing.

Alternatively, the transporter activity could be modulated directly by protein degradation, or indirectly by altering the proton motive force available via the regulation of H^+-ATPase.

Moreover, the differences between the diurnal expression of the *STP1* gene and its subsequent transport activity may be a result of abiotic factors modulating the *STP1* transcript level or STP1 transport activity during the uptake assay. There is an appreciably change in the *STP1* transcript level in plantlets at the beginning of the uptake experiment and that seen at the end (figure 4.3.3). In plantlets subjected to the uptake assay conditions, the *STP1* transcripts are higher during the light phase, and lower during the dark phase relative to that seen in untreated plantlets (harvested at the beginning of the experiment). The diurnal expression pattern of the *STP1* gene at the end of the uptake experiment more closely resembles the 3-OMG uptake pattern by plantlets entrained to a L12 h:D12 h regime. It is possible that the increase in *STP1* transcripts in the light phase is a result of mechano-stimulation, water induction, stress or lack of oxygen. Whereas, the decrease in *STP1* transcripts during the dark phase is likely to be an effect of light upon the plantlets subjected to the uptake assay. Furthermore, the change of mRNA levels between the 2 sets of plantlets occurs rapidly, within 1 h. This is indicative of regulation by mRNA stability, which would explain the rapid turnover of dark-accumulated *STP1* transcripts.

Further experimentation is required to understand the mechanisms controlling the synthesis and activity of the STP1 transporter. An STP1 antibody or an STP1-tagged protein could be used to characterise the post-translational regulation of the STP1 protein, and perhaps the modulation of the STP1 transporter activity by sugar and/or light directly.

To conclude, a distinct pattern of STP1 synthesis and activity is observed in both the

light and dark phases of plantlets entrained to a diurnal cycle, as well as, in plantlets subsequently transferred to continuous environmental conditions. Moreover, the *STP1* gene expression and 3-OMG transport activity is different in the shoots compared to that in the roots. This is consistent with the regulation of genes involved in carbohydrate partitioning, distribution and utilisation by the 24 h biological clock, which is used to co-ordinate the biological processes within the plant during a normal diurnal cycle. In a manner similar to that observed in light-grown plantlets, the *STP1* transcript levels are modulated by light, glucose and mechanical stimulation. However, the extent to which the *STP1* gene responds to such factors varies throughout the diurnal cycle. These results are discussed in terms of STP1 transporter function in chapter 5.

CHAPTER FIVE: General Discussion

5.1 Regulation of the synthesis and activity of the STP1 transporter

The allocation of carbohydrates throughout the plant is controlled by the action of sucrose and hexose transporters. Consequently, the regulation of such transporters determines the distribution of sugars to different cell-types. Sugar can in turn function as a signalling molecule regulating several key biological processes, for example, photosynthesis, starch biosynthesis, cell cycle and defence mechanisms. Therefore, it is anticipated that a regulatory network to connect sugar transport with other signal transduction pathways exists *in planta*. This is supported by the identification of three inter-linking regulatory factors that modulate the expression of the *STP1* hexose transporter gene. The expression of the *STP1* gene is regulated developmentally (Sherson *et al.,* 2000), as well as by sugar, light and mechanical stimulation. Furthermore, these abiotic factors appear to regulate the *STP1* gene in a co-ordinated manner. For example, the induction of *STP1* transcript levels by mechanical stimulation is apparently greater in plantlets incubated in the light relative to that in the dark (section 3.3.6) and this induction is completely repressed by exogenous glucose (section 4.5.2).

The expression of the *STP1* gene can be repressed by a number of sugars including D-glucose and sucrose (section 3.3; Hemmann, 2000). Henmann (2000) also used glucose analogues (3-OMG and 2DOG) to investigate the importance of glucose transport and subsequent phosphorylation in mediating a glucose response. Glucose repression of *STP1* transcripts was seen upon treatment of plantlets with exogenous 2DOG, but not 3-OMG. This suggests that the *STP1* gene responds to sugars in a hexokinase-dependent manner.

In humans and Yeast, hormone signal transduction pathways control the regulation of sugar transport and carbon metabolism; for example, insulin stimulates the uptake and metabolism of glucose (Hellmann *et al.,* 2000). In plants, hexose uptake

is increased by cytokinin treatment (Ehness and Roitsch, 1997). The inter-play of sugar mediated gene expression with phytohormone signalling pathways, such as ABA and ethylene, is well documented (Finkelstein and Gibson, 2001; Gazzarrini and McCourt, 2001). However, the expression of the monosaccharide transporter *STP1* gene appears to be responsive to glucose in an ABA-independent manner. The response of the *STP1* gene to glucose in mutants defective in ABA synthesis and ABA response resembled that seen in wild-type (section 3.3.5). The regulation of other sugar-responsive genes, such as, *CAB*, *PC* and *RBCS* are reportedly impaired in these mutants (Dijkwel *et al.,* 1996 and 1997). Future experiments employing exogenous ABA, for example, are needed to confirm *STP1* gene regulation does not interact with ABA signalling. Moreover, future experiments to investigate the importance of phytohormone signalling upon the synthesis and activity of the STP1 transporter would be interesting. For example, monitoring the *STP1* transcript level in *etr*, *gai* and *aux* mutant plantlets, which are defective in ethylene, gibberellin, and auxin hormone responses respectively.

In recent years, research into the sugar signalling transduction pathway in plants, has focused on the analysis of mutants defective in sugar perception or sugar responses. However, the screening conditions used to isolate such mutants employed the application of extremely high sugar concentrations (100-300 mM) to seedlings. Thus, it is possible that mutants isolated in this way are defective in stress response, due to the non-physiological conditions employed. In the future, the *STP1* promoter::luciferase reporter construct could be used in a similar mutant screening programme, but concentrations of glucose an order of magnitude lower could be used. By exploiting the sensitive sugar response of the *STP1* gene, mutants' defective in potential glucose perception, glucose signal transduction, or possibly the glucose-mediated regulation of transcription, post-transcriptional stability, post-translational processing or transport activity of the STP1 transporter

could be identified. Moreover, as well as investigating the sugar-sensing pathway, such mutants may also reveal the impact of altered monosaccharide transport upon the allocation of sugars throughout the plant.

The negative feedback of STP1 synthesis and/or activity by its substrates results in a reduced rate of glucose uptake by both shoot and root tissue, and possibly reduced uptake of other monosaccharides. The regulation of STP1 transport activity can, therefore, control the uptake of glucose by the cell, and consequently determine the availability of glucose present in the cell to transduce intracellular sugar signalling.

Sugar transport plays an important role in the partitioning of carbohydrates between source and sink tissues. Furthermore, most of the genes implicated in the partitioning of carbohydrates are diurnally regulated; an important shift in carbohydrate metabolism occurs between the photosynthetic production of sugars in the light and starch metabolism and the subsequent utilisation of sugars during the night (Koch, 1996; Geiger *et al.,* 2000). This is consistent with the complex expression pattern of the *STP1* gene and glucose transport activity upon entrainment of plantlets to a diurnal growth regime. For example, the diurnal expression of *STP1* gene in mutants with altered sugar metabolism and/or content (*sex4*, *pgm1* and *det3*) is different to that observed in wild-type (section 4.5.3). This suggests that either carbon metabolism, flux of sugars, flux of signalling molecules e.g. Ca^{2+}, or the resulting sugar content within the cell could potentially regulate the expression of the *STP1* gene.

5.2 Potential physiological function of the STP1 monosaccharide transporter

The gene expression and transport activity of STP1 is seen in both the shoots and the roots (section 4.2); the *STP1* gene is also highly expressed in developing seeds (Sherson *et al.,* 2000). It is possible that the STP1 monosaccharide transporter has distinct roles in different organs. For example, in sink organs, such as developing seeds and roots, STP1 may predominantly function in the provision of hexoses for metabolism and growth; whereas in source leaves STP1 may function in the retrieval of hexoses that may leak from cells.

It is possible that the STP1 transporter may have other functions *in planta,* which are consistent with the regulatory factors that modulate *STP1* gene expression. The *STP1* gene may be classified as a dark-inducible (*din*) and a touch-inducible (*TCH*) gene. Other genes that are regulated in a similar manner, include those encoding endo-glucanase (EGases; Nicol and Hofte, 1998), xyloglucan endoglycosylases (XET; Xu *et al.,* 1995) and glucohydrolase (Roulin *et al.,* 2002). These enzymes are thought to function in the modification of glucan polymers in the cell wall. The rearrangement, transfer and hydrolysis of the carbohydrate matrix is required for cell elongation to occur (Cosgrove, 2000).

In *Arabidopsis,* there are at least 10 EGase gene families. One class is ethylene inducible and its expression correlates with massive cell wall degradation during fruit ripening and leave abscission (Brummell *et al.,* 1994). Another class is also involved in hydrolytic processes during the rearrangement of glucans in cell walls, which may play a role in cell expansion by promoting cell wall loosening (Nicol and Hofte, 1998). XETs are cell wall modifying enzymes that are thought to alter the properties of the cell wall during plant development, or in response to an environmental

stimulus, via transglycolysation or hydrolysis of xyloglucan polymers. In *Arabidopsis*, the expression of an *XET* gene was induced by mechanical stimulation, darkness, auxin and brassinosteroid (BR) treatment (Braam *et al.,* 1997). β-D-glucan glucohydrolyases (β-gluc) catalyse the hydrolysis of β-D-glycosidic bonds, releasing single, non-reducing terminal glucose residues from both glucan polymers and oligoglucosides derived from the carbohydrate matrix of the cell wall. In barley, the expression of a *β-gluc* gene is induced 10-fold by darkness (Roulin *et al.*, 2002; Leah *et al.,* 1995). Taken together, these observations provide evidence for the potential liberation of monosaccharides from the cell wall during the dark. If this is the case, then in plantlets transferred to darkness or mechanically stimulated both *STP1* transcripts and the level of sugars derived from the cell wall increases. Furthermore, the induction of the *STP1* gene correlates with the induction of genes involved in cell wall elongation or modification. It is possible that the STP1 transporter may function in the salvage of monosaccharides liberated from the cell wall during the modification of the cell wall carbohydrate matrix, for use in metabolism or storage. Moreover, this is consistent with the *in vivo* localisation of *STP1*-driven luciferase expression in areas of cell expansion, such as, young expanding leaves, lateral roots and the elongation zone of the root (figure 3.6.1 and 3.6.2).

The synthesis and activity of the STP1 transporter occurs in all the plant tissue analysed in this study. It is possible that due to the diverse locations of the STP1 transporter throughout the plant, STP1 has more than one physiological role. For example, STP1 may function in the provision of sugars to sink organs, such as the roots, for use in metabolism and growth; whereas in expanding leaves STP1 may have a role in the salvage of cell-wall-derived sugars. Furthermore, the synthesis and activity of the STP1 transporter appears to be integrated with sugar-sensing and

other signalling pathways. The complexity of regulation of the STP1 transporter is consistent with a number of different roles *in vivo*.

5.3 Direction of future work

The potential role for STP1 in the salvage of cell-wall-derived sugars could be investigated by analysing the gene expression and transport activity of STP1 in mutants with defective cell expansion. The short hypocotyl *Korrigan* (*Kor*) mutant is defective in the synthesis of an EGase, and displays a reduced cell elongation/expansion phenotype (Nicol and Hofte, 1998). If the synthesis and/or activity of the STP1 transporter is co-ordinated with cell wall modification and expansion, then the regulation of STP1 in such mutants will also be affected. The *det3* mutant that was used in this study, also displays a short hypocotyl phenotype and is defective in cell elongation (Schumacher *et al.,* 1999). The expression of the *STP1* gene in *det3* mutant plantlets is different from that seen in wild-type (section 4.5.3). This is consistent with the potential co-ordinated regulation of the STP1 transporter with that of cell wall modification/expansion.

Moreover, many of the genes that encode the cell wall modifying enzymes described above (and in section 1.11) are regulated by phytohormones, such as brassinosteroids, auxin and gibberellic acid. The *STP1* gene is regulated in a similar manner to such genes in response to darkness and mechanical stimulation; therefore it may be worthwhile to investigate the *STP1* gene response to phytohormones. This could be investigated by monitoring the gene expression and transport activity of the STP1 transporter in plantlets treated with exogenous hormones, or in plantlets defective in hormone biosynthesis or response pathways. For example, the *bri1* mutant is insensitive to BR and the *ga4-1* mutant is defective

in GA biosynthesis. The regulation of *STP1* gene expression is as wild-type in ABA synthetic and ABA response mutants (section 3.3.5).

Furthermore, by examining the cell wall properties, cell expansion and sugar content of the cytosol and apoplast of the *stp1* mutant relative to wild-type, the effect of defective STP1 activity upon 'sugar salvage' could be investigated. Expansive growth of a plant cell can be measured by monitoring the changes in turgor pressure. A 'pressure probe cell' can be used to measure turgor pressure of individual plant cells *in situ* (Francoise Marga, University of Louisana website, accessed on the 03/07/02, www.ucs.louisiana.edu/~fsm2405/probe.html). Cytosolic (Zeeman *et al.,* 1998) and apoplastic sugars can be extracted, separated and quantified by HPLC. Differences in the apoplastic sugar content of the *stp1* mutant and wild-type plantlets would be consistent with a role STP1 transporter function in the salvage of sugars derived from the cell wall.

The *stp1* mutant is defective in the principal monosaccharide transporter of *Arabidopsis* (section 4.3.3; Sherson *et al.,* 2000). However, *stp1* mutant plants appear to grow and develop as wild-type; this suggests that STP1 is not essential for 'normal' growth and development to occur. Although, it may be that in a growth room or greenhouse the conditions are not stressful or competitive enough for any discernable difference between wild-type and mutant to be observed. The STP family consists of at least 14 transporters; it is possible that in the absence of the STP1 transporter, other STPs fulfil its role.

It would be interesting to investigate this further, perhaps by comparing the 'fitness' of the *stp1* mutant relative to wild-type. Following the growth of an equal quantity of wild-type and mutant plants together through several generations, the relative 'fitness' of each plant may be determined by analysing the ratio of each plant in the

final population. For example, the fittest plant would represent a greater proportion of the final population. In tobacco, an antisense hexose-transporter transgenic line displays an apparently marked phenotype relative to wild-type (unpublished work by Delrot *et al.,* 2000). This suggests that, in tobacco at least, monosaccharide transport is necessary for normal plant growth and development to occur. The requirement for monosaccharide transport in other plant species could also be investigated. In species, such as *Vicia faba,* the provision of hexoses is essential for the normal seed development to occur; it would be interesting to see if a monosaccharide transporter null mutant was compromised in such development.

Moreover, there is an ever increasing number of *Arabidopsis* mutants available through various seedbanks. Therefore, it is now possible to generate double and triplet mutants. Crossing the *stp1* mutant plant with other mutants, such as those defective in cell wall elongation, phytohormone signalling, or sugar transport, the importance of STP1 activity may be revealed. Perhaps multiple *STP* mutants may result in a marked phenotype, possibly revealing distinct roles for monosaccharide transport during development and growth. Multigenic mutant plants could be used to investigate cross-talk between signalling pathways e.g. ABA and sugar; or the possible co-ordination of cellular processes e.g. cell expansion and sugar salvage from cell wall turnover.

CHAPTER SIX: References

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